Dating Death:
Forensic Taphonomy and the Postmortem Interval

Thesis submitted for the Degree of Doctor of Philosophy

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Abstract

Determining the postmortem interval (PMI) remains one of the most important but challenging factors to establish in a suspicious death investigation. Unfortunately, as time passes current methods lose accuracy and only allow investigators to approximate how long ago death occurred. Bodies interred in clandestine graves prove particularly challenging due to an abundance of variables that need to be taken into consideration. Due to the problems associated with determining the PMI of buried remains this study will utilise macroscopic, microscopic, molecular, chemical and microbiological analyses to systematically document the decompositional changes to human hair and porcine cartilage and bone in a burial environment. The aim was to correlate decompositional changes with time and develop new methods for estimating the PMI of remains found in this context.

Whole trotters (from which the cartilage was harvested) exhibited decompositional changes including darkening of the dermis, skin slippage, liquefaction of soft tissues and complete skeletonisation. The decompositional changes to cartilage included a loss of cartilage covering articular facets, changes in colour and texture, formation of orthorhombic crystals, a change in surface pH and colonisation by bacteria. The bacteria found on the cartilage surface were in close proximity to the crystals and when cultured on a B-41 medium were found to precipitate crystals of the same morphology and chemical composition to those found on the cartilage surface. Three species of bacteria (*Acinetobacter calcoaceticus, Acinetobacter iwoffii* and *Grimontia hollisae*) were identified based on gas chromatography–mass spectrometry (GC-MS) of their fatty acids and one species (*Comamonas* sp.) was identified by DNA analysis. Formation of crystals on goat and cow cartilage proved that this was not a porcine specific phenomenon. Human hair exhibited a gradual degradation over time but this was dependent on the characteristics of the burial environment. Decompositional changes included colonisation by fungi, erosions to the cortical surface and formation of tunnels and breaks to the hair shaft. Two fungal species (*Aspergillus fumigatus* and *Penicillium* sp.) were identified based on DNA analysis of fungal ribosomal (rDNA) internally transcribed spacer (ITS) regions. The *Penicillium* sp. was linked with fungal tunnelling of hair. Bone exhibited little modification over time but changes were observed. These included a change in colour of the cortical surface, a change in colour and gradual loss of bone marrow and erosions, cracking and flaking of the cortical bone. Fungi were found to colonise both the bone marrow and bone surface. Whole piglets were buried to document the

time period taken to reach skeletonisation. This data was used as a correction factor and combined with the bone results to give an overall time period for the decomposition changes observed.

The results of this study suggest that the decompositional changes to cartilage could be used to determine the postmortem interval of buried remains. However, the degradation of hair and bone was too variable to be of use in this context.

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Only one more stage to go and then this mighty piece of work can take pride of place on my bookshelf.

Any omissions are entirely my fault and I apologise wholeheartedly for overlooking you.

Dedication

This thesis is dedicated

To my Mother and Father,

Lyn and Les

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Chapter 1: Introduction

We owe respect to the living: to the dead we owe only the truth.
Voltaire, Première lettre sur Oedipe

1.1 Introduction

Determination of a postmortem interval (PMI) is integral to the disciplines of forensic medicine and forensic science, referring to the amount of time that has passed since death. Determining the PMI is a question that is often posed to the forensic scientist (Sledzik, 1998), in particular forensic anthropologists (e.g. Ubelaker and Houck, 2002), pathologists (e.g. Pounder, 1995), entomologists (e.g. Goff *et al.*, 1991), botanists and palynologists (e.g. Buchan and Anderson, 2001) and the mycologist (van de Voorde and Van Dijck, 1982). However, an understanding of the postmortem decomposition process is integral to establishing the PMI (Campobassa *et al.*, 2001). Establishing a PMI can help in the identification of human remains by narrowing down the list of missing persons and establish the final movements of a deceased individual (Willey, 1987). Additionally, documentation of the time of death is paramount on official forms such as death certificates and insurance forms, administrative details regarding funeral proceedings, legal claims related to property, debts and personal estates (Burton, 1974, Beaumont, 2007). Komar and Buikstra (2008), add further examples such as, verifying or refuting alibis, assessing variability in toxicological results, establishing cases of neglect in penal institutions or care homes, and assessing health and safety concerns for investigators, i.e. viability of pathogens.

Sledzik (1998) states that one way to imagine decomposition is as a linear progression, and at points along this line different scientific methods are employed to determine how much time has elapsed since death. However, decomposition rates are notably variable due to anatomical variation between persons (Knight, 2004) and environmental conditions (Sledzik, 1998, Knight, 2004) resulting in difficulties in ascribing an accurate PMI value. Often, at best, only an estimation can be presented.

This research will document the postmortem changes to hair, cartilage and bone by applying macroscopic, microscopic, molecular, microbiological and chemical methods. The postmortem changes to these tissues will be used to develop new methods to estimate the postmortem interval of buried remains, thus contributing to the field of forensic science. Out of the three tissues to be investigated hair and bone are the most widely documented attracting research from numerous academic areas including archaeology, palaeontology and forensic science and covering multitude of phenomena (Petraco *et al.*, 1988, Baumgartner *et al.*, 1989, Pete *et al.*, 1989, Schoeninger, 1992, Stiner *et al.*, 1995, Millard, 1996, Bell, 1996, O'Connell, 1999, Tafaro, 2000 and Ambrose, 2003).

The thesis is presented as seven self-contained chapters. The first chapter focuses on presenting a systematic literature evaluation of taphonomy and its role within forensic science. This chapter details the decomposition processes explaining algor mortis, hypostasis, rigor mortis, autolysis, putrefaction, skeletonisation, diagenesis and a small section focused on the decomposition of foetal remains. Additionally, this chapter will briefly explain special circumstances whereby a body undergoes preservation. This chapter will allow for an understanding of the decomposition processes and the factors which influence or retard them. Chapter one progresses to detail the influence of burial on the decomposition process and the role fungi play as saprophytic organisms in a forensic context. This chapter concludes by discussing the current methods utilised to determine the postmortem interval. Methods including assessment of body cooling, forensic entomology, potassium levels in the vitreous humour, carbon 14 analysis, and forensic botany are discussed.

The second chapter presents a basic characterisation (consisting of pH and XRF) of the locales and soil type in which the experiments were conducted and explains the importance of understanding the burial environment in forensic interment studies. The third, fourth and fifth chapters provide details for the degradation of hair, cartilage and bone respectively. Each of these chapters presents a review of the current published literature focusing on the tissue composition and its decomposition. These chapters continue to explain the methods used and the results gained during the course of this research. Each of these chapters concludes with a critical evaluation of the research findings and places them within the context of the current literature. Chapter six details the decomposition of whole piglets in a burial environment with the aim of determining the duration taken for complete skeletonisation to occur. Finally, chapter seven presents a general discussion and combines the findings and research outcomes for all of the individual chapters that make up this thesis. This chapter details the major limitations and the scope for future investigation and presents a new method for determining the postmortem interval. Included is a paper detailing the decomposition of cartilage published within the Journal of Forensic and Legal Medicine.

The aims and objectives of this research include the following:

Aim 1:

To systematically evaluate the current literature focusing on taphonomy, decomposition and the methods used to determine the postmortem interval.

Objectives:

1. To conduct an extensive literature review focusing on taphonomy in a forensic context, postmortem decomposition, forensic medicine and current and emerging methods used to determine the postmortem interval.

Aim 2:

Does cartilage follow a predicable decomposition sequence and can this information be used to determine the postmortem interval?

Objectives:

1. To conduct a literature review detailing the composition and postmortem decomposition of cartilage.
2. To conduct burials of porcine trotters to act as human analogues.
3. To execute systematic analysis of postmortem cartilage using macroscopic and microscopic methods.
4. To conduct detailed analysis of surface crystals known to develop on cartilage postmortem to discover their identity and ontogeny.
5. To identify any bacterial species found inhabiting cartilage postmortem using DNA species specification and GC-MS analysis of fatty acids.

Aim 3:

Can a decomposition sequence be developed for hair in a burial location and can this information be used to determine the postmortem interval?

Objectives:

1. To conduct a literature review focusing on the composition of hair and how it decomposes by weathering and microbiological activity.
2. To conduct burial of human hair.
3. To execute light microscopy and scanning electron microscopy of hair to observe decompositional changes.
4. To conduct microbiological analysis on hair to focus specifically on fungal species that colonise the hair shaft.
5. To subject the colonising fungi to DNA analysis for species specification.
6. To determine if fungal colonisation follows a predictable pattern and if this pattern can be utilised to determine a postmortem interval.

Aim 4:

To establish the time taken for skeletonisation of interred whole piglets.

Objectives:

1. To inter whole piglets in shallow graves.
2. To establish the time taken for the buried piglets to reach skeletonisation.
3. To use this data as a correction factor for the cartilage extracted from amputated porcine trotters.

1.2 Taphonomy and its Forensic Implications

The term taphonomy, meaning the 'law of burial', was first introduced by Efremov (1940) and derives from '*taphos*' (meaning burial) and '*nomos*' (meaning law) (Nawrocki, 1996). It includes expertise from academic fields such as; palaeontology, archaeology, and more recently, mycology, botany, entomology and forensic science (Carter and Tibbet, 2003, Forbes, 2008). Traditionally taphonomy, defined as the systematic study of fossil preservation and preservational processes (Brett, 1986), encompasses the study of mortality, decay, disarticulation, transport, burial, bioturbation, reworking and time averaging of fossil assemblages, depositional environments, diagenesis, and completeness of the fossil record (Martin, 2000). Martin (2000) presents an exhaustive list but it must be remembered that not all organic remains become fossilised over time. Brett (1986), splits taphonomy into two spheres, biostratinomy and fossil diagenesis. Within biostratinomy he classifies biogenic processes that break down remains, namely transportation, disarticulation, fragmentation and corrosion. Within fossil diagenesis he details skeletal dissolution, sediment filling and mineralisation as examples. For an in depth discussions of these processes please refer to Brett (1986). Actualistic studies are readily conducted in taphonomy (see Andrews, 1995) using present day patterns and processes to help understand the past (Kowalewski and Labarbera, 2004). Data collection in actualistic taphonomy can be conducted via three ways; a) in the field by making direct observations, b) data collection by field experimentation, c) data collection in the laboratory by controlling variables (Kowalewski and Labarbera, 2004). Each of these methods are valuable scientific tools, contributing new knowledge to the discipline. Of note is that the methods presented can be interlinked, for example, data collection within the laboratory and field (points C and B) will ultimately help understand observations within the field (point A). The method of data collection is dependent on the research questions posed.

'Forensic taphonomy' is concerned with understanding decomposition processes and the factors that influence them, as relevant in a legal context (Tibbett and Carter, 2008). Please refer to chapter 1.1 for further information relating to the legal context. Just as Brett (1986) divides traditional taphonomy in to two sub-fields, taphonomy within a forensic context can also be subdivided. Forensic taphonomy has two branches, biotaphonomy, which is directly concerned with the corpse postmortem and geotaphonomy, which is concerned with the act of burial itself, together with the interactions between the environment and the corpse, see table

1.1 (adapted from Nawrocki, 1996). Haglund and Sorg (1997), world leaders in forensic taphonomy and editors of two critically acclaimed forensic taphonomy texts, further explain that taphonomy has a multidisciplinary approach taking into consideration biological, cultural and geological factors.

Table 1.1: The branches of forensic taphonomy and factors that are found within each, after Nawrocki (1996).

Branches of Forensic Taphonomy and Subsequent Factors	
Biotaphonomy	Environmental Factors, **Abiotic** (climate) **Biotic** (animals/plants)
	Individual Factors (body size, age at death).
	Cultural Factors (embalming, violent trauma).
Geotaphonomy	Disturbance to the soil as the grave is dug.
	Production of tool marks in the formation of the grave.
	Disruption of botanical species in the graves vicinity.
	Foot prints left in the bottom of the grave.
	Changes in soil pH due to decomposition.

As forensic scientists are concerned with the recent dead (compared to archaeology or palaeontology), both soft and hard tissue modifications are of significant interest (Haglund and Sorg 1997). Ubelaker (1997) an anthropologist with decades of forensic experience, discusses taphonomic applications in forensic anthropology and details the aspects of animal scavenging, weathering patterns, and estimating the PMI, environmental reconstruction, sequence of postmortem events and pseudo-trauma. Ubelaker states that an understanding of

each of these aspects is vital in forensic anthropology, demonstrating that taphonomy encompasses more than just the decomposition process.

Forensic taphonomy has a somewhat chequered past. According to Haglund and Sorg (1997), in 1987 taphonomy was eliminated as a key word from the Journal of Forensic Sciences, most probably as a result of loss of interest or popularity. However, taphonomy, as a key word, was reinstated but only appeared a total of six times between 1989 and volume six of 1993 (Haglund, 1991, Haglund and Sorg, 1997). This suggests a continued lack of research interest or application of taphonomy to forensic investigations. In one important forensic anthropology texts of recent times "The Human Skeleton in Forensic Medicine" (Krogman and Işcan, 1986), taphonomy is only mentioned three times but paradoxically the text does recommend taphonomic analysis for human remains (Haglund and Sorg, 1997). This omission of discussion regarding taphonomy could reflect the authors' personal research interests which do not include taphonomy but rather focus on physical anthropology and dental anatomy. However, the recommendation of taphonomic analysis illustrates an appreciation of the subject and the value such analysis may offer to forensic investigations. Haglund and Sorg (1997), present three plausible reasons why there has been little research into forensic taphonomy;

1. The lack of availability of human remains for research. The general view in society highlights death as an 'emotionally charged issue' (Haglund, 1991).
2. Limited involvement of forensic anthropologists in death investigations.
3. Limited awareness of the use of forensic taphonomy in death investigations.

The second point is further elaborated by Black (2000), who sent out a questionnaire to all police forces in the UK of which 75% responded. Out of this 75%, 98% have never required the assistance of a forensic osteologist. Black (2000) stated that most of the time such work is carried out by a forensic pathologist. It must be emphasised that Black's research is based on events within the United Kingdom and that circumstances in other countries may differ. Although Black is discussing the role of a forensic osteologist, forensic osteology and forensic anthropology are similar disciplines, with Black stating that forensic osteology is a small branch of forensic anthropology and describes how the osteologist tends to be concerned with the initial phases of the investigation of a corpse or skeleton before the wider discipline of forensic anthropology is implemented. Also, Haglund (1991), explains that the

majority of bodies are found while still fresh, for example, persons who have died in medical institutions or road traffic accidents, and thus do not require the expertise of an anthropologist who specialises in taphonomy. Furthermore, specific facilities that execute taphonomic research on human cadavers exist in only a few countries (e.g. USA). The UK for example, does not allow such research. Presently, much of the published literature uses case studies and reviews to assess taphonomic characteristics and document various processes (Komar and Buikstra, 2008). Thus, forensic taphonomy is largely descriptive rather than based on systematic scientific analysis and associated theory. Consequently, it relies upon, as with the wider discipline of taphonomy, actualistic approaches and the theory of uniformitarianism (Komar and Buikstra, 2008). However, the theory of uniformitarianism, i.e. processes that occur today are believed to be the same that occurred in the past, remains untested in the forensic arena (Komar and Buikstra, 2008).

In their seminal work, Haglund and Sorg (1997), present one of the most exhaustive definitions of forensic taphonomy to date:

"Forensic taphonomy refers to the use of taphonomic models, approaches, and analyses in forensic contexts to estimate the time since death, reconstruct the circumstances before and after deposition, and discriminate the products of human behaviour from those created by the earth's biological, physical, chemical, and geological subsystems".

Forensic taphonomy has five main goals; a) to estimate time since death, b) differentiate between human and non-human bone modification, c) understand transport of remains,

d) assess variables resulting in differing bone preservation, and, e) reconstruct perimortem events. A detailed description of these aspects can be found in Komar and Buikstra (2008).

1.3 Postmortem Phenomena

Postmortem decomposition is reported to begin immediately after death (Dent *et al.,* 2004) when there is a cessation in biological functions maintaining integrity of cells (Miller, 2002). Decomposition begins in as little as four minutes after vital signs have ceased (Vass, 2001, Vass *et al.,* 2002). Knight (2004), distinguishes between somatic and cellular death and states that when somatic death occurs, cellular death may be incomplete with some cells still capable of movement (Knight, 2004). This is corroborated by Gill-King (1997), who explains that, in western culture, death is seen as an event but in actuality death is not an event but a process, as not all cells 'die' at once. He further discusses the physical and chemical

constraints of decomposition explaining that all bodies undergoing decomposition undergo these processes, but the temperature of the immediate environment governs how fast or slow these processes occur. Furthermore, he explains how water, pH, and partial pressure of oxygen can have an impact on the decomposition process.

Clark *et al.,* (1997), explain that one of the first signs of death is pallor of the skin resulting from the termination of blood circulation. When oxygenated blood is restricted from the skin, it is observed to be a characteristic grey colour which can occur in light skinned people in as little as 15 minutes after death (Clark *et al.,* 1997). At the time of the emergence of this body discolouration, the body's muscles relax, which may result in faecal discharge or regurgitation of gastric contents (Clark *et al.,* 1997). Clark *et al.,* (1997), further report early postmortem changes in the eye such as "tache noir sclerotique" which is drying of the corneal epithelium caused by relaxation of the eye muscles, typically manifested as a black or darkened line that horizontally crosses the eye.

During the initial stages of decomposition numerous processes occur that fall within the realm of forensic pathology. Forensic pathology is a discipline specialising in the analysis of 'fresh' soft tissues and the forensic pathologist regularly has to establish the earlier postmortem intervals when the body exhibits minimal decompositional changes (Hughes-Collier, 2001).

1.3.1 Algor Mortis, Body Cooling, Chill of Death

During life, metabolic processes help maintain a constant temperature in homeothermic organisms. However, after death, the body will begin to lose its heat as these processes stop (Clark *et al.,* 1997). Burton (1974), gives this process a dramatic name, 'the chill of death'. Heat will be lost mainly via radiation and convection with evaporation being an issue if the subject is wet (Pounder, 1995). Cooling will occur until the body reaches ambient temperature in a process known as algor mortis or body cooling (Pounder, 1995, Knight, 2004). There are numerous factors which will influence how quickly a body will cool, namely, initial body temperature (illness can elevate the bodies temperature), body size or dimensions (thin people cool faster than large), clothing or coverings (clothed individuals will cool slower than naked) and air movement (Pounder, 1995). Furthermore, the medium in which the body is situated will also have an effect on cooling rates, for example a body recovered from water as discussed by numerous forensic pathologists (Burton, 1974, Pounder, 1995, Knight, 2004).

Further details relating to algor mortis and its application to PMI estimation will be discussed later.

1.3.2 Hypostasis, Lividity, Livor Mortis, Darkening of Death

Hypostasis is also referred to as 'lucidity', 'staining' or 'cogitation' (Knight, 2004), but is also given the dramatic name of 'the darkening of death' by Burton (1974). Knight gives an excellent description of the processes of hypostasis explaining that it occurs when circulation stops and gravity acts on the stagnant blood bringing it down to the lowest levels of the body, which is corroborated by Wonder (2001, p.7). This process of settling is able to occur because, within 30-60 minutes, the blood becomes incoagulable due to the release of fibrinolysins, and thus remains in a liquid form (Pounder, 1995, Clark *et al.*, 1997). Pounder (1995) explains that the bluish tinge to this blood is not due to a pathological process such as cyanosis but due to continued oxygen disassociation and a reflux of deoxygenated venous blood into the blood vessels. Interestingly, under low ambient temperature, lividity can adopt a bright pink colouration due to resaturation of haemoglobin with oxygen (Bohnert *et al.*, 1999). Lividity can present within 20-30 minutes postmortem, first as small patches which will coalesce over time and become fixed after 10-12 hours (Pounder, 1995). Amendt *et al*. (2004) state that lividity is generally evident after two hours and is fixed after four-six hours after death due to the fat in the dermis solidifying in the capillaries.

1.3.3 Rigor Mortis, Stiffening of Death

One of the most documented and well known processes of decomposition is rigor mortis, or as Burton (1974) explains, 'the stiffening of death'. This stage is widely discussed in the forensic literature. Rigor mortis is evident under favourable conditions in about 2-6 hours following death (Gill-King, 1997, Clark *et al.*, 1997, Amendt *et al.*, 2004, Jackson and Jackson, 2004, p.329). The process is initially apparent in the eyelids, jaw and neck (Clark *et al.*, 1997, Amendt *et al.*, 2004, and Jackson, 2004, p.329) and progresses down the body with the arms, trunk and then legs being affected. The whole body is in a rigor state at approximately 12-24 hours postmortem (Clark *et al.*, 1997). The process of rigor mortis is not permanent with a post rigor flaccid state occurring at approximately 24-36 hours postmortem (Clark *et al.*, 1997, Jackson and Jackson, 2004, p.329). Gill-King (1997) reports that rigor can

last as long as 84 hours. It can be seen from these two authors alone that there is no agreement about how long the rigor state lasts with these differences likely due to differences in temperature (as Gill-King originates from the USA and Jackson and Jackson the UK) or differences in personal experience. The process of flaccidity begins in the same sequence as rigor began (eyes, jaw, neck etc.). Once rigor is evident it is possible to 'break' it by manual manipulation. A study conducted by Krompecher *et al* (2007), showed that rigor can in fact be re-established after it is broken if the initial process was not complete. However, Krompecher *et al* (2007), found that the re-established rigidity was weaker after the breaking. The presented hypothesis states that rigidity occurs progressively, one muscle fibre at a time (which is dependent on the fibres metabolism). The fewer the number of fibres, the easier rigor mortis can become re-instated (Krompecher *et al.,* 2007). These experiments were conducted on rats that had been killed by carbon dioxide inhalation. Consequently, it would be of interest to see if these results can be applied to human cadavers. Unfortunately, such research could not occur in the UK due to numerous restrictions.

Gill-King (1997), explains that the sarcoplasmic reticulum of muscle cells begins to disorganise and calcium ions flood into the sarcomere, which consists of alternating parallel protein filaments of actin and myosin. The calcium ions unblock the binding site of the actin molecule allowing the two molecules to bind via a cross arm (also known as a cross bridge) extending from the myosin (Gill-King, 1997). When this cross arm is pulled back the actin molecule is pulled along the myosin, consequently the overall muscle tissue is shortened and becomes firm and rigid. As the Adenosine triphosphate (ATP) levels in the body have decreased, this link between the actin and myosin cannot be chemically reversed, thus rigor is induced. As already mentioned rigor desists after a passage of time. This is believed to be caused by an occurrence of a structural change in the myofibrils (Gill-King, 1997). It is believed that proteolytic enzymes may be involved in the detachment of actin and myosin (Gill-King, 1997). Gill-King further continues his description stating that rigor is influenced by temperature. In warmer environments, rigor occurs slowly and is often delayed, while colder environments promote and often prolong rigor. He also explains that if the organism was in an excited metabolic state prior to death then rigor may occur rapidly after death, probably due to greater amounts of lactic acid present in the muscle. Furthermore, rigor mortis may occur faster in people suffering from a fever (Clark *et al.,* 1997). These factors need to be addressed prior to the analysing the state of rigor.

Varetto and Curto (2005), observed that rigor mortis is more persistent at lower temperatures, explaining that full rigor mortis can be seen in bodies that have been refrigerated for 10-16 days.

1.4 Postmortem Tissue Changes

1.4.1 Autolysis

The process of decomposition begins with autolysis also referred to as self-digestion (Vass, 2001). Autolysis is governed by endogenous and liberated enzymes (Knight, 2004, Tsokos, 2006) that naturally occur in one's own body. It is an aseptic phenomenon (Powers, 2005). Vass (2001) gives a particularly good biochemical, introductory explanation of the processes leading up to autolysis. He explains that as cells become oxygen (O_2) depleted, a build-up of carbon dioxide (CO_2) occurs as a natural metabolite of cellular processes (which, as already stated continue briefly in some cells after death has occurred). As a consequence of this increase in CO_2 the pH of the body becomes acidic and as natural detoxification processes stop after death, waste products (e.g. lactic acid) begin to build up. Cell membranes are not maintained (as during life) and falling ATP levels (ATP in life aids in biosynthesis and repair of cellular membranes) results in loss of membrane structure. Furthermore, due to the decreasing ATP levels, transport through cellular membranes is unregulated and the cell may begin to swell (Gill-King, 1997). Additionally, enzymes naturally found compartmentalised in the body begin to dissolve cell membranes which consequently lyse and release cellular contents into the body. It is widely reported that autolysis will first become apparent in the most metabolically active regions of the body first (for example the liver) (Gill-King, 1997, Vass, 2001). Gill-King (1997), explains this process in more detail. He explains that as cells become O_2 depleted the cellular biochemistry begins to fail, essentially the ATP (Adenosine Triphosphate) levels drop as the mitochondrial electron transport becomes disrupted. He further explains that when the circulation stops and there is a decrease in pH and an increase in waste products and cellular biochemistry switches from a metabolic pathway to a fermentative one (Gill-King, 1997). In this fermentative pathway, pyruvic acid is converted to lactic acid anaerobically. With the falling levels of ATP, cellular membranes begin to lose their structure as membrane fatty acids are lost via oxidation and not replaced. Furthermore,

active membrane transport cannot occur (Gill-King, 1997). When cell membranes cease to function, enzymes that are normally compartmentalised are liberated and become activated under the decreased pH levels and further degrade the cell membrane. Eventually cells will become detached from each other in a process called necrosis. Gill-King (1997), gives details of the order of tissue decomposition explaining that it occurs first in the tissues and organs concerned with digestion, and last in the connective tissues.

The released nutrients liberated by the breakdown of cellular material will later act as a nutritional source for micro-organisms. Macroscopically observable features of autolysis include skin slippage. This phenomenon occurs when the liberated hydrolytic enzymes digest cells at the dermal-epidermal junction (Clark *et al.,* 1997). Consequently, the epidermis can slough off of the body. Furthermore, hair and nails can also become detached. Postmortem bullae can also be observed. These are fluid filled blisters located on the skin (Clark *et al.,* 1997). These can rupture upon movement and can provide an obvious risk to any investigative personnel through exposure to pathogenic agents (see Demiryürek *et al.,* 2002 for a discussion of infectious agents in cadavers). Internally, autolysis can manifest as a dough like consistency to the soft tissues (Clark *et al.,* 1997). Haemolysis will also be evident due to breakdown of red blood cells which consequently causes a marbled appearance on the external surface of the body (Clark *et al.,* 1997).

1.4.2 Putrefaction

After the body is depleted of oxygen it becomes an anaerobic environment. This environment is essential for initial putrefaction. Putrefaction is ultimately the destruction of the body's soft tissues by anaerobic micro-organisms (Pounder, 1995, Vass, 2001, Vass *et al.,* 2002, Statheropoulos *et al.,* 2004). In essence this process involves the breaking down of large molecules into smaller, simpler structures (Dent *et al.,* 2004). Putrefaction requires the presence of bacteria, which, in the initial stages, come from the body's own colonies in the intestinal tract, and respiratory systems (Pounder, 1995, Dent *et al.,* 2004). The species involved are anaerobic and consist of bacilli, coliforms, micrococci, and diphtheroids (Pounder, 1995). In the later stages of decomposition, when the structural integrity of the body is compromised and additional openings occur, bacteria may immigrate from the surrounding environment (Campobasso *et al.,* 2001), especially if soil is present (Dent *et al.,* 2004). Furthermore, sites of injury (trauma) can act as a passageway for external micro-

organisms (Campobasso *et al.*, 2001). This may allow for external bacterial participation in the putrefactive process early on, in essence speeding up the processes.

Bacterially produced hydrogen sulphide reacts with haemoglobin to form sulfhaemoglobin resulting in a green discolouration (Amendt *et al.,* 2004). Putrefaction first becomes apparent in the right iliac fossa, which is where the caecum is situated superficially beneath the dermis (Knight, 2004). Pounder (1995), states that a bacterial infection prior to death will speed up the onset of putrefaction. He also states that in the UK, the degree of putrefaction seen in 24 hours in summer may require 10-14 days in winter because of the difference in average temperature (Pounder, 1995). This observation probably stems from Pounder's professional experience as a forensic pathologist as it is difficult to find corroborating evidence in the UK literature.

Gas formation in the body, from the breakdown of tissues, will become obvious in the area of the abdominal cavities, visually apparent as detention of the abdomen and scrotum. The face will also bloat resulting in protrusion of the eyeballs and tongue (Knight, 2004). There have been cases of postmortem childbirth where a foetus is expelled from the body by the build-up of gases (Knight, 2004, Schulz *et al.,* 2005). Furthermore, this build-up of gases will also result in purging of fluids from various orifices. The major gases that accumulate are nitrogen, methane, hydrogen sulphide and ammonia (Amendt *et al.,* 2004, Campobasso *et al.,* 2001) and carbon dioxide (Statheropoulos *et al.*, 2004). The pressure that these gases create may cause splitting of the skin, resulting in postmortem injuries as discussed by Vass (2001).

After several weeks, dependent on environmental conditions, the colour of the body will change to dark green, almost black colours (Knight, 2004). Warm environments provide adequate conditions for bacterial growth and consequently will speed up putrefaction of body tissues (Spennemann and Franke, 1995). Under favourable conditions (with easy access) the body will be home to a host of insect species (Goff, 1991, Byrd and Castner, 2001, Campobasso *et al.*, 2001, Shean *et al.,* 1993, and Matoba *et al.,* 2007). Kintz *et al.,* (1990), explain that when undergoing putrefaction a body will become a target for necrophagous insects (attracted by odours released), with Calliphorid larvae being the most prevalent.

Internal organs will putrefy at different rates depending on their anatomy and physiology; the pancreas for example will degrade quickly after death whereas the prostate and uterus may still be recognisable in contemporary skeletal material (Knight, 2004). Obese people can provide a particular challenge to investigators. When fat decomposes it may liquefy and fill

the body cavities (Knight, 2004). Obese people are also documented to decompose faster compared to people with lower amounts of adipose tissue (Dent *et al.*, 2004).

In an open environment, the body will become oxygenated and the level of decomposition will increase due to the free access of air (Dent *et al.*, 2004). Aerobic microorganisms such as fungi may be found in association with this stage of decomposition (Dent *et al.*, 2004)

Eventually the body's cavity will begin to collapse and the soft tissues will liquefy leaving only the most durable biologic components. Nicholson (1996), states that once soft tissues have become absent, biological decomposition may be supplemented by chemical degradation. However, in addition to Nicholson's (1996) statement, biological decomposition can continue despite loss of soft tissues. These processes will be discussed later.

1.4.3 Skeletonisation

Skeletonisation is defined by Dent *et al.*, (2004), as the removal of soft tissue from bone. Consequently after the majority of soft tissues have degraded, ligaments, tendons and periosteal tags will remain adhered to the skeleton (Knight, 2004) this is documented to take approximately 12-18 months in a temperate climate (Knight, 2004). For surface remains, decomposed soft tissue will be washed down into the soil by rainfall (Janaway, 1996, Dent *et al.*, 2004). If a body is located outside, then large predators will act as scavengers feeding on the remaining soft tissues and disarticulate what remains of the body (Haglund, 1991).

1.4.4 Diagenesis

When the soft tissues have decomposed and the skeleton is exposed, bone can undergo a series of processes collectively known as diagenesis. This phase of the decomposition process tends to attract research from the archaeological and anthropological communities as opposed to pathologists. The organic and mineral constituents of bone are degraded by the action of microbial agents, plants, soil and groundwater (Wedl, 1864, Piepenbrink, 1986, 1989, Cox and Bell, 1999, Turner-Walker, 2008 and Stodder, 2008). The collagen phase of bone is degraded by the action of bacteria, in particular the bacterial collagenases they produce. These enzymes break the protein-mineral bond to peptides reducing them to amino acids (Nawrocki, 1995). The inorganic portion of bone is degraded through the loss of hydroxyapatite (HAP) by weathering which ultimately weakens the integrity of the bone resulting in disintegration

(Hare, 1976). Furthermore, HAP can be structurally altered (Hedges and Millard, 1995). Bone diagenesis will be discussed in detail in chapter 5.

1.5 Foetal decomposition

Details relating to foetal decomposition remain scarce in the literature. Perhaps, due to the emotive response to deceased sub adults and the understandable unwillingness for parents to allow full autopsies, thorough data pertaining to foetal decomposition cannot be effectively gathered. In utero a deceased foetus will not follow normal decompositional processes, instead foetal tissues will soften in a process known as maceration from the Latin '*macerare*' meaning, to soften by soaking, (Moore, 2007). Autolysis via internal proteolytic enzymes also contributes to the softening of the tissues (Moore, 2007). Pounder (1995), elaborates stating that the autolysis is aseptic without bacterial putrefaction due to lack of an established microbial community in the foetal gastrointestinal tract. Initial signs of maceration include skin slippage resulting in exposure of the underlying dermis (Moore, 2007). 24 hours after intrauterine death (IUD) bullae start to form between the dermis and epidermis with the epidermis losing its structure after three-four days (Pounder, 1995, Moore, 2007). The organs and associated connective tissues begin to change colour after 48 hours due to the haemolysis of red blood cells. Furthermore, autolysis of the connective tissues results in a laxity of the joints (Moore, 2007) and the limbs become flaccid (Pounder, 1995). Maroun and Graem (2005), conducted a study looking at the differences in body parameters between nonmacerated and macerated foetuses and concluded that the weight of the liver, thymus, spleen, lungs, kidneys and adrenals decreased with an increase in maceration, the overall foetal weight however did not differ. It was also found that the body length slightly increased in the macerated subjects. As time progresses cranial bones become separated from the dura and periosteum (Moore, 2007) and the skull becomes mobile (Pounder, 1995). After 7-10 days the foetus will often exhibit a brown colouration and loss of fluid will result in shrinkage of tissues (Moore, 2007). Once delivered, it is essential that any postmortem examination is conducted immediately as putrefaction will begin promptly (Pounder, 1995).

1.6 Exceptions to the general decomposition process: postmortem preservation

This section will discuss instances whereby the decomposition process is interrupted or substituted in cases of preservation. It is not the aim of this thesis to present the minutiae of such phenomena, but rather to provide this information as it may be a requested to determine the PMI of preserved human remains.

1.6.1 Lithopaedion

In exceptional circumstances a foetus can undergo processes resulting in the formation of a lithopaedion or 'stone child' (*lithos*: stone, *paedion*: child) (Odom *et al.*, 2006, Mishra *et al.*, 2007). Lithopaedion, the calcification of a foetus, is a consequence of abnormal pregnancy whereby the placenta is able to re-establish its blood supply after ruptured tubal or uterine pregnancy, occurring within the peritoneal cavity (Chang *et al.*, 2001). Several conditions are required for a lithopaedion to form; a) the foetus should survive for over three months, b) sterility of the foetus with no bacterial involvement, c) the death of the foetus and consequent calcification remains undiagnosed or misdiagnosed, d) and, conditions favourable for calcium deposition. Küchenmeister (1881), classified three categories of lithopaedion, a) lithokelyphos, where the foetal membranes calcify and the foetus degrades within them, b) lithokelyphopedion, where both the foetus and membranes exhibit a degree of calcification, c) true lithopedion, where the foetus is calcified but the membranes are not. Further studies of lithopaedion are presented by Griffith (1930), Storck *et al.*, (1939), Chase (1968), Lachman *et al.*, (2001), Hag *et al.*, (2002), and Jesinger *et al.*, (2010).

1.6.2 Mummification

Mummification can prevent putrefaction and occurs under favourable conditions such as a dry environment (Jit *et al.*, 2001, Knight, 2004). Mummification occurs in any desiccatory environment (Forbes *et al.*, 2005c), including in freezing conditions (Knight, 2004). Anthropogenic mummification occurred thousands of years ago and was a practice conducted by the ancient Egyptians (Sandison, 1963, Klys *et al.*, 1999, Kaup *et al.*, 2003, Knight, 2004). Natural mummification occurs with a moving air current which helps to desiccate or dry out tissues helping keep putrefaction at bay (Knight, 2004, Powers, 2005).

Knight (2004), details the morphological characteristics of mummification as desiccation and brittleness of the skin, which is stretched tightly across anatomical prominences such as the cheekbones, chin, costal margin and pelvis (Knight, 2004). He also gives reference to discolouration of the skin and colonisation by moulds. The discolouration of the skin is reported to be caused by subjection to slow oxidative processes (Powers, 2005). An examination of mummified subjects will also reveal shrunken and desiccated internal organs along with a darkening of the dermis (Jit *et al.*, 2001, Forbes *et al.*, 2005c). The process of mummification may also result in a decrease in stature of the overall body, due to a loss of water from cartilage (Jit *et al.*, 2001). The time required for mummification may take as little as a few weeks to several months depending on the surrounding environment (Jit *et al.*, 2001). Mummification in a contemporary context can occur in closed environments such as attics, cupboards, or under floorboards as discussed by Jit *et al.*, (2001) and Beaumont (2007). Further information is present by Hubbert (1974) and Wu (1988).

After several years, the mummified body will begin to disintegrate via the action of moulds and the leathery tissues will start to split. Eventually, this disintegration will leave only the skeleton with small pieces of adhering mummified tissues (Knight, 2004).

1.6.3 Adipocere formation

Adipocere is also referred to as saponification (Vass, 2001) and is the conversion of body fat into a soapy, waxy, lipid mixture (Forbes *et al.*, 2002). Adipocere can form from any body fat (Takatori, 2001). It has been studied since the 18th Century and is regarded as process of natural corpse preservation (O'Brien and Kuehner, 2007). This conversion of body fat to lipid mixture occurs best under humid, anaerobic conditions and under advantageous situations it can appear partially after a few weeks. However, formation covering the whole corpse can take several years (Forbes *et al.*, 2002). Adipocere is caused by the hydrogenation and hydrolysis of saturated fatty acids and was once thought to occur in mainly aqueous environment. However, this is now known to be incorrect as the body's own water content is suitable (Forbes *et al.*, 2005). Forbes *et al.*, (2005), state that adipocere can form even in dry soils and water tight environments, (Nushida *et al.*, 2008). Decomposition is inhibited due to the release of fatty acids that retard the growth of putrefactive bacteria (Pounder, 1995)

Adipocere will, eventually, break down (Forbes, 2008a). When adipocere is exposed to oxygen it will begin to decompose. A study by Fründ and Schoenen (2009), showed that this occurred within a time span of 10 years. The authors suggest this is due to microbiota with

evidence pointing to fungi as adipocere decomposing agents. For further details of adipocere formation please refer to Takatori, (1996, 2001), Stuart *et al.,* (2000), Dent *et al.,* (2004), Forbes *et al.,* (2004, 2005a, 2005c, 2008a), Kumar *et al.,* (2009) Notter *et al.,* (2009), Fründ and Schoenen (2009), Bull *et al.,* (2009).

1.6.4 Preservation by frozen conditions

Bodies may undergo freezing either deliberately or due to presence in a cold environment (Stokes *et al.,* 2009). Freezing causes the withdrawal of water from cells leading to a decrease in diffusion between cells thus inhibiting chemical reactions (Partmann, 1963, Stokes *et al.,* 2009). When a frozen body is thawed it may be difficult to determine the postmortem interval (Schäfer and Kaufmann, 1999). During freezing, up to 80% of water contained within a cell is drawn into the interstitial channels forming ice crystals at -5 °C (Stokes *et al.,* 2009). At -10 to -15°C cellular cytoplasm will freeze. Cells react to freezing in two ways; either by shrinking or freezing internally (Schäfer and Kaufmann, 1999).

Connections holding cells together can become disrupted during freezing and may not reform once thawed, often leading to softening and liquefaction of tissues with weak intercellular junctions (Schäfer and Kaufmann, 1999). This will have an axiomatic impact on the decomposition process.

Freezing may affect decomposition rates as discussed by Stokes *et al.,* (2009). Frozen animals on the ground exhibited decomposition from the outside with preservation of the internal organs. This is reported as a consequence of retardation of enteric growth of microorganisms, and mechanical disruption of skin, connective tissues and joints (Micozzi, 1997). Conversely, fresh corpses decompose from the inside under anaerobic conditions (Micozzi, 1986). Some authors claim that freezing may have a biocidal effect on enteric organisms (Micozzi, 1997) and freezing may also alter enzymes thus inhibiting their functional conformation (Stokes *et al.,* 2009). Micozzi (1997), states that generally, temperatures below 12°C greatly reduce bacterial reproduction, and at temperatures below 5°C bacterial growth ceases completely. Freezing studies were conducted on bone by Tersigni (2007), who concluded that freezing has little impact on bone microstructure despite cracking around the Haversian canals. However, a possible limitation for this study lies within the experimental design in utilising bone sections rather than intact whole bones. Using sections would result in direct exposure of the bone's

internal microstructure which may not normally occur during the freezing of intact bodies. For further details regarding freezing, please see Notman *et al.*, (1987), Tabata *et al.*, (2000).

1.6.5 Bog bodies

Peat cut from peat bogs has been used as a fuel source for centuries (Brothwell and Gill-Robinson, 2002). During the cutting of peat in northern Europe preserved human remains have been discovered. These are termed bog bodies and can span a long history, from prehistoric to modern. Peat bogs offer a remarkable environment for preservation which is often acidic, anaerobic (Omar *et al.,* 1989) and microbially sterile (Connolly, 1985). Bogs are unbalanced ecosystems producing more plant matter than they decompose (Sibley, 1998). Consequently, the excessive build-up of this organic matter prevents oxygenation of deep water. The presence of sphagnum moss results in the extraction of calcium and magnesium ions and the liberation of hydrogen ions (Sibley, 1998). The presence of a polysaccharide called sphagnan derived from sphagnum moss (van der Plicht *et al.,* 2004) is vital in the preservation of bog bodies. Sphagnan results in the formation of humic acids and also reacts with enzymes produced by bacteria consequently rendering them inactive and causing a tanning of the dermis (Painter, 1995). Under such conditions bodies exhibit good preservation of hair and skin (Omar, *et al.,* 1989). For further details please see Stankiewicz *et al.*, (1997).

1.7 Buried Bodies

So far the basis of decomposition has been discussed. However, this study aims to observe the decomposition and degradation of hair, cartilage and bone in a grave environment, consequently it is beneficial to discuss how a body decomposes in this type of setting.

There is a general rule of thumb utilised within the forensic area known as Casper's Dictum. This rule stipulates that (at exposure to the same temperature) putrefaction in air after one week is equivalent to two weeks of immersion in water which is, in turn equivalent to eight weeks of burial (Camps and Cameron, 1971).

Burial is a common method for disposal of deceased persons, this is usually under legal circumstances. However, on occasion, burial in a more nefarious context may be encountered (Gaudry, 2010). This research aims to assit investigators in the latter context.

Unfortunately, at present there is little literature focusing on decomposition in a burial environment (Miller, 2002), and what is present is often incomplete (Dent *et al.,* 2004). When

a body is introduced into a burial (without a coffin) the process of decomposition is ultimately influenced by the soil (Dent *et al.*, 2004). The burial environment encompasses the biological, geological and chemical conditions that are present in a particular location (Forbes *et al.*, 2005c).

It is widely known that burial is reported to slow down the rate of decomposition (Sledzik, 1998, Miller, 2002, Knight, 2004, Byers, 2005, p118). Sledzik (1998) and Miller (2002), explain that this is due to limited access for insects and scavengers. However, further considerations, such as sunlight should be taken into account. Deep burials are protected from solar energy by soil and thus remain at a cool temperature (Miller, 2002). Solar energy from the sun heats up the earth, increasing the temperature of superficial soil horizons. Such increases in temperature can influence decompositional processes (Carter and Tibbett, 2006).

The idea that bodies decompose slower in graves was demonstrated by Rodriguez and Bass (1985) who interred cadavers in graves spanning 1 ft to 4 ft in depth for durations of 1 month – 1 year, they found that decomposition occurs slower in burials of greater depth. Rodriguez and Bass' research remains landmark in that the study utilised human cadavers. However, in what seems to be a common theme within forensic taphonomic research, the study suffered from a small sample size. Knight (2004), explains that if a body is interred immediately after death then putrefaction may never actually occur. However, if the body has already started to decompose when the interment occurs then decomposition may take place at a slower rate (Knight, 2004). The soil pH is important, high alkaline or low acidic soils will inhibit bacterial growth and consequently decomposition may be hindered or even cease (Forbes *et al.*, 2005a). Furthermore, the oxygen content of the burial environment will also effect decomposition, aerobic bacteria and fungi that use the corpse as a food source require sufficient oxygen content to survive (Forbes *et al.*, 2005a), thus anaerobic environments tend to promote cadaveric preservation.

Janaway (1996) explains that when in a grave environment a body is subjected to numerous variables and each burial is, in essence, unique (Janaway, 1996). This statement demonstrates the difficulty in assessing the PMI of buried remains and illustrates why continual research is crucial. Millar (2002), explains that a body in a shallow clandestine grave (restricted to the topsoil and subsoil), will decompose in a similar way to that of a body exposed to the open air, but the process is somewhat slower.

Once a body is placed in a soil environment it will be subjected to microbial activity. The majority of these organisms will be saprophytic (Nicholson, 1996).

1.8 Fungal Growth at Sites of Decomposition and Burials

Goff (2010) explains that the outer surface of the human body is made of dead material which is shed during everyday activities, consequently adhering spores and moulds are lost. However, postmortem this dead material (*stratum corneum*) is not shed resulting in fungi colonising the body.

Fungi acquire nutrition via two main routes: as saprophytic or parasitic organisms. The saprophytic fungi are important in the decomposition of remains to break large molecules down into their simple constituents (Webster, 1980). The occurrence of fungal growth at sites of carcass decomposition has received much attention from the Japanese scientific community compared to other geographic regions such as the UK or USA. Three particular fungal species have been of interest and documeneted, *Hebeloma vinsophyllum*, (Sagara, 1976, Takayama and Sagara, 1981, Fukiharu, Yokoyama and Oba, 2000), *Hebeloma radicosum*, (Kuroyanagi, Honda, Yoshimi and Sagara, 1982) and *Laccaria proxima* (Sagara, 1981). The carcasses studied were from various organisms (this lack of consistency could be regarded as a limitation to these studies) but the general consensus stipulates that fungal growth occurs at sites of liberated ammonia released during the decomposition process. Hopkins *et al.*, (2000) discuss elevation of ammonia in grave soil during decomposition.

More recently, this fungal phenomenon has been explored by Tibbett and Carter (2003), who present a study detailing the fungi that mark woodland graves. They detail two chemoecological groups of fungi, the ammonia fungi (AF) and the post-putrefaction fungi (PPF). These species of fungi are stated as being associated with decompositional by-products (Tibbett and Carter, 2003). The aim of the study was to determine whether sporophores may be useful in locating graves. This was indeed proved possible, as post-putrefaction fungi can be markers of soil disturbance and decomposition. The authors also state that a time since burial (TSB) estimate could be obtained by analysing the early and late stage fruiting species. These findings could have future value in the fields of forensic archaeology and taphonomy. However, there are issues that need further exploration, for example, the temperature, soil chemistry, and treatment of soils with fertilizers, as little is known regarding these effects on fungal colonisation. These factors require better understanding before the application of fungi in forensic contexts becomes more reliable. Tibbett and Carter (2003), also state that the temporal relationship between cadaver decomposition and fungal fruiting is little understood.

1.9 Current Methods for Attributing PMI

Determination of the postmortem interval remains one of the most challenging aspects of any suspicious death investigation (Pinheiro, 2006). In the early stages of decomposition this finding is usually best determined by the forensic pathologist or the forensic entomologist (Klepinger, 2006). As the postmortem interval progresses and the remains in question become too compromised, the estimation of the time elapsed since death may become the purview of the anthropologist (Klepinger, 2006). Unfortunately, present methods used to determine the postmortem interval lose accuracy as the postmortem interval increases (e.g. Klepinger, 2006). For bodies that have decomposed and are just comprised of bones, determining a PMI is a frequent problem and of great importance (Castellano *et al.*, 1984).

1.9.1 Assessment of body cooling (algor mortis)

Cooling of the body is a physical process (Henßge and Madea, 2004), and measurements of the core body temperature will be taken upon the discovery of a body. This temperature can be applied to numerous methods for assessing the postmortem interval such as the nomogram method (Hessenge, 1988), which is regarded as the leading method for determining the postmortem interval in the early stages and can be routinely found in forensic pathology texts (e.g. Knight, 2004). This method seems preferred as the effect of clothing, water and air can be taken into account. Forensic pathologists routinely use the body temperature to estimate the PMI as it is seen as the most useful method for the first 24 hours after death (Pounder, 1995) as on average the body loses 1.5˚F an hour (Clark *et al.*, 1997). However, there is somewhat of a discrepancy regarding the sampling area for this analysis. Henβge and Madea (2004), state that rectal and central brain temperatures are the only temperatures relevant to this method. Conversely, Baccino *et al.*, (1996) provide other sampling sites for the assessment of body temperature include the outer ear, but this is disregarded by Catanese *et al.*, (2010) who state that the ear temperature is unacceptable for use during this analysis. There is no obvious reason for this disagreement over sampling sites but personal experience or preference could be postulated. For a detailed history and literature review of the use of body cooling for estimating time since death please refer to Kaliszan *et al.*, (2009). For further methods for determining time since death from body cooling please refer to al-Alousi *et al.*, (2002a, 2002b).

1.9.2 Assessment of hypostasis (livor mortis)

In 1996 a method presented by Vanezis and Trujillo (1996), focused on measuring hypostasis using a colorimeter. This method proved successful but was, unfortunately, limited by a small sample size. Recently, the consensus states that hypostasis plays a minor role in determining the postmortem interval (Henβge and Madea, 2004). Knight (2004), elaborates that the process of hypostasis has no relevance to the PMI as its onset is variable, and it may not be visible in infants, the senile or persons with anaemia (Knight, 2004). Interestingly, Bhat *et al.*, (2006), explain that the use of lividity is not reliable in bodies exposed to cold environments. However, they seem to only score the appearance and fixation of lividity which, if judging from statements of the previous authors, seems inadequate. The problem of determining the postmortem interval by use of lividity is still being investigated, with a recent publication, Usumoto *et al.*, (2010), presenting the use of spectrophotometric analysis and the formulation of mathematical equations. They describe the precision of this method within +/- 4.76 hours. Although this study proved successful, it requires peer testing in order to validate the findings presented.

1.9.3 Assessment of rigor mortis

The use of rigor mortis to establish a PMI is reported to be highly unreliable (Jackson and Jackson 2004, p.329). This is because the onset of rigor is highly variable and congruent with temperature and immediate antemortem activity. Knight (2004) states that rigor mortis is a poor indicator of the postmortem interval. He presents a spot check list stating the following (Knight, 2004):

- If the body is warm to the touch but flaccid, it has been dead for less than 3 hours.
- If the body is warm to the touch but stiff or rigid, it has been dead 3-8 hours.
- If the body is cold to the touch and is stiff, the body has been dead 8-36 hours.
- Finally, if the body is cold and flaccid, it has been dead for more than 36 hours.

Krompecher *et al.*, (2007) disagree by stating that rigor mortis is routinely used to estimate time since death, although no details relating to accuracy of the methodology are presented. Using the above spot check list may provide a viable presumptive test but relying on rigor as

the sole determinant of a postmortem interval would be inadvisable. Similarly to the assessment of hypostasis, Henßge and Madea (2004), state that rigor is of minor significance regarding reliable information of the time of death interval.

1.9.4 The use of insects in determining the postmortem interval (Forensic entomology)

One of the most heavily researched disciplines used in the estimation of time since death is that of forensic entomology (e.g. Goff *et al.,* 1991). In recent years there has been a renewed interest in forensic entomology and how it can be applied to determine PMI estimates (Goff *et al.,* 1991). Due to this interest the subject has received a wealth of research, making it one of the most accurate methods available (Buchan and Anderson, 2001). The decomposing corpse provides a rich substrate for a wide variety of organisms (Dadour and Harvey, 2008), and the estimation of the PMI in this context is based on the knowledge of insect succession on a corpse (Dadour and Harvey, 2008). Diptera, or true flies, will be the first insects to arrive at a corpse, followed by other insects that are attracted to the corpse in its different stages of decomposition e.g. coleoptera or beetles, (Buchan and Anderson, 2001, Jackson and Jackson, 2004, p.326).

When a body is found the entomological evidence must be collected and identified which requires advanced knowledge in insect taxonomy. The level of insect development must be noted as soon as possible (Buchan and Anderson, 2001). Temperature and other weather data are vital to help calculating the time required for an insect to reach a particular stage of development (Williams, 1984) which will help in determining how much time has passed since the insect egg was oviposited on the corpse (Haskell *et al.,* 1997). This obviously means that only a minimum time internal can be estimated. For further information on forensic entomology please refer to Kintz *et al.,* 1990, Shean *et al.,* 1993, Goff, 2000, Campobasso *et al.,* 2001, Gennard, 2007, Huntington *et al.,* 2007.

1.9.5 Assessment of fungal colonisation on cadavers

Recently, fungal colonisation on human remains has been investigated to see if a postmortem interval could be determined (Ishii *et al.,* 2006). Ishii *et al.,* (2006), state that to their knowledge their paper was the first to describe in detail the fungi obtained from a corpse. In fact, this is incorrect as an earlier study by van de Voorde and Van Dijck (1982), discusses the fungi cultured from a deceased 57 year old female. In their study, van de Voorde and Van Dijck were able to determine a postmortem period of (between) 11-23 days based on fungal growth in a laboratory setting. This analysis proved surprisingly accurate, as when apprehended, the suspect admitted murder 18 days prior to the body's discovery.

Hitosugi *et al.,* (2006), conducted analysis on a cadaver of a 71 year old male discovered at the bottom of a well and were able to determine a postmortem interval of 10 days. However, the articles by Ishii *et al.,* (2006) and Hitosugi *et al.,* (2006) have received criticism within the forensic community, especially focusing on the lack of discussion relating to the rates of growth on cadavers and factors that may influence such growth (Menezes *et al.,* 2008), and, in the case of Ishii *et al.,* (2006), after discussing the use of fungi on cadavers, be able to present a crude postmortem interval estimation for the two cases presented which was lacking in their conclusion (Menezes *et al.,* 2007).

A recent publication by Sidrim *et al*., (2009), identified *Aspergillus*, *Penicillium* and *Candida* as fungi growing on decomposing bodies in Brazil. This paper provided no correlation between the observation of these fungi and the determination of the postmortem interval but does suggest a possible difference of fungal species occurring at the bloating, putrefactive and skeletonisation stages of decomposition.

The use of fungi to determine the postmortem interval has potential but at present is too subjective and the variables relating to growth and influencing factors are too poorly understood to hold against legal scrutiny. The findings discussed above demonstrate that the use of fungi in forensic contexts may, in the future, become a valid technique. However, at the moment, the data gathered provides potential for further research.

1.9.6 Analysis of potassium concentration in the vitreous humour and other biochemical methods

The vitreous humour is a transparent gelatinous substance that occupies the posterior four fifths of the eyeball (Prasad, 2003). As the retina degrades, potassium is leached into the

vitreous humour, due to the failure of the sodium potassium pump (Prasad, 2003). Needle aspiration of the vitreous humour occurs during an autopsy and analysis is conducted to determine the potassium concentration. The aspiration must be conducted accurately to avoid inclusion of retinal cell fragments which may distort analytical values (Knight, 2004). Studies conclude that there is a linear elevation in the vitreous potassium level as the postmortem interval increases, which is of use in determining the PMI (Prasad, 2003). This is emphasised by a study conducted by Garg *et al.*, (2004), who confirm an increase in potassium over time, but also that the levels of potassium measured bilaterally in the same individual exhibited no significant difference. They also found that potassium levels in the eye were elevated in burn cases indicating that temperature influences potassium levels. However, this technique has not gained practical acceptance within the forensic community.

Numerous methods exist for determining the PMI in the early period but unfortunately have not gained practical relevance since they were only of academic interest (Henßge and Madea 2007). Madea (2005), concludes that the use of thanatochemistry - the analysis of chemical changes that occur after death (Hirvonen et. al., 1997) is limited. Another interesting method presented by Sabucedo and Furton (2003), details PMI estimation based on the analysis of cardiac Troponin I postmortem and is described as being useful for periods of 0-5 days postmortem.

Currently, there are numerous methods available for establishing the PMI in the later stages although most of these methods lack (high) accuracy. The longer the time between death and analysis, the less exact and precise the estimation of the postmortem interval will be (Garg *et al.*, 2004, Bhat *et al.*, 2006). Introna *et al.*, (1999), detail that a rough PMI estimation from skeletal remains can be conducted by observing the presence of connective tissues, this however is very tentative and should not be the sole analysis conducted. Below is a brief overview of the main methods used for determining the PMI of bodies in the later postmortem period.

1.9.7 The use of botany in determining the PMI

Plants found in association with human remains may prove of use in determining the postmortem interval but the method is rarely utilised, (Buchan and Anderson, 2001).

Botanical species that contain growth rings (perennial plants) can be utilised, the plant can be sectioned and the growth rings counted (dendrochronology). The growth rings are annually produced and can provide an estimate of the minimum time the plant has been in association with the remains (Wiley and Heilman, 1987). Vanezis *et al.*, (1978), give details of how damage to plant roots can help in determining when a grave was dug. For details relating to the method and application of botany to estimating PMI please refer to Quatrehomme *et al.*, (1997) and Courtin and Fairgrieve (2004). Similarly to forensic entomology, an obvious limitation with the application of botany to PMI estimates is that an actual accurate PMI cannot be established, only a minimum time range.

1.9.8 The assessment of C^{14} and other isotopes

Radiocarbon dating is a widely accepted and routinely used technique in archaeology for dating skeletal material (Forbes, 2008b). Carbon-14 (^{14}C) is constantly formed via the interaction of the earth's atmospheric nitrogen with cosmic radiation the product of which is dispersed through the atmosphere as $^{14}CO_2$. ^{14}C becomes part of the food chain by incorporation into plant matter (Gunn, 2009, p.39, Hua, 2009). When an organism dies incorporation of ^{14}C stops and its abundance begins to decrease allowing the level of ^{14}C in the body to be measured against the atmospheric levels, indicating how long an organism has been dead (Ubelaker and Houck, 2002, Gunn, 2009, p.39, Hua, 2009). Assessment of dates up to 50,000 years before present can be determined (Tuniz *et al.*, 2004). A further application of ^{14}C dating involves the bomb peak. During the 1950's and 1960's testing of nuclear weapons resulted in an increase of ^{14}C in the atmosphere (Pollard, 1996, Forbes, 2008b, Forbes and Nugent, 2009) and in the northern hemisphere, ^{14}C levels were nearly 100% higher than they were at the pre bomb level (Zoppi *et al.*, 2004). The amount of ^{14}C in the atmosphere began to decrease after the signing of the 1963 Nuclear Test Ban Treaty (Wild *et al.*, 2000, Tuniz *et al.*, 2004, Forbes, 2008b), presently standing at 10% greater than the pre-bomb level due to exchange of ^{14}C with the biosphere and oceans (Zoppi *et al.*, 2004). These ^{14}C values can be used to produce a calibration curve which is useful for radiocarbon dating young samples (Wild *et al.*, 2000). Bomb-pulse dating has successfully been applied forensically in instances of war crimes (Tuniz *et al.*, 2004). Taylor *et al.*, (1989), state that ^{14}C dating of modern or recent bone can place the bone in one of several categories: Pre 1650, 1650 to 1950, and post 1950, with the latter indicating forensic interest. Advances in ^{14}C

dating in relation to accelerator mass spectrometry (AMS) has allowed for the analysis of samples in the sub-milligram weight (Tuniz *et al.*, 2004). This holds particular importance in forensic cases where preservation of evidence is crucial. Interestingly, radiocarbon dating can be applied to cremated bone (Olsen *et al.*, 2008).

Swift (1998) and Swift *et al.*, (2001) explain the use of ^{210}Polonium and ^{210}Lead (Po^{210} and Pb^{210}) in determining PMI. Pb^{210} is absorbed in the body through inhalation and consumption of food and water and uptake during life is stated as being relatively constant. It is incorporated into bone by replacing calcium within the osteological matrix (Forbes, 2008b). Pb^{210} has a half-life of 22 years and decays to bismuth 210 (Bi^{210}) to form polonium 210 (Po^{210}) (Forbes, 2008b). An equilibrium between Po^{210} and Pb^{210} due to differences in their half-lives is eventually reached and remains stable during life. However, upon death, the ratio between Po^{210} and Pb^{210} distorts (Forbes, 2004b). Consequently, the analysis of Pb^{210} in osseous material can be used to determine a PMI (Forbes, 2008b). Interestingly, this method was previously used to date sediment (Swift, 1998). However, several limitations are described including the effect of diagenesis, expense, time limitations and a lack of knowledge regarding the background of the samples analysed relating to diet and smoking (Swift, 1998, Swift *et al.*, 2001). The latter limitations pose obvious concerns with unidentified human remains where such lifestyle preferences are unknown. Similar methodologies are also presented by Neis *et al.*, (1999), who explain the use of Strontium 90 for determining PMI. They state that the method is simple, reliable and useful for determining whether somebody died before or after the 50th year of the last century (Neis *et al.*, 1999). At present it seems that this method can be used as a presumptive test and should be combined with other methods to produce a more accurate PMI estimation.

1.9.9 The use of luminol and nitrogen content for estimating the PMI

Luminol, for a long time, has been used by crime scene examiners and forensic biologists to test for the presence of blood (Creamer and Buck, 2009, Ramsthaler *et al.*, 2009). The use of luminol has now been applied to estimate the PMI from skeletal elements. The durable nature of bone protects haemoglobin, which for as long as the structural integrity of bone remains solid, stays contained in a stable environment (Creamer and Buck, 2009). Introna *et al.*, (1999), used luminol to test for blood residue in femora, luminol was applied to ground compact bone and the level of the reaction intensity was assessed. They concluded that there

is a possible quantitative relationship between the increase in PMI and a decrease in luminescence intensity. However, the authors state more samples should be analysed with a closer ranging PMI to document statistical significance (Introna *et al.*, 1999). In a further study, Creamer and Buck (2009) found that the use of luminol is promising but, as with the study conducted by Introna *et al.*, (1999), the study suffers from a small sample size. Creamer and Buck (2009), state that this method can help in distinguishing between forensic (<100 years old) and historic (>100 years old) remains. Interestingly, the authors state the breakdown of haemoglobin is consistent throughout the skeleton (Creamer and Buck, 2009). In a further study Ramsthaler *et al.*, (2009), concluded that chemiluminesence testing is not adequate as a sole method for distinguishing between recent and non-recent remains due to the observance of false positive and false negative results.

Jarvis (1997), presents a method of using a macro Kjeldahl technique for determining deposition time of human remains from nitrogen levels in long bones of bodies buried in coffins for 29-90 years. This conclusion showed that nitrogen levels decreased over time, but some anomalous results show this method is not always consistent. However, this method is in agreement with earlier findings by Knight and Lauder (1967, 1969), who also document decreasing nitrogen levels with the increasing postmortem interval.

1.9.10 Assessment of DNA degradation

Immediately after death, biological and environmental processes begin to degrade DNA (Buchan and Anderson, 2001). In this first instance the process is governed by autolysis and putrefaction which act to degrade cellular material consequently causing exposure of DNA to nucleases (Parsons and Weedn, 1997). Eventually microfauna such as bacteria will also contribute to the degradation of DNA (Buchan and Anderson, 2001). As the DNA degrades the size of the DNA molecule decreases, and the rate at which this occurs could be of value in assessing the postmortem interval (Buchan and Anderson, 2001). Environmental factors such as temperature and humidity have proven to result in DNA degradation (Perry *et al.*, 1988). It is suggested that the interaction between bone and its environment has a more detrimental effect on bone than the time elapsed since death (Buchan and Anderson, 2001, Parsons and Weedn, 1997). Bearing this in mind an important assessment of the immediate environment is recommended before the degradation of DNA can be applied to determine the PMI.

1.9.11 Assessment of the changes in bone microstructure

Structural changes to bone have only recently been applied to determine PMI. Consequently there is little research in this area (Buchan and Anderson, 2001). Structural changes are not only environmentally dependent but are also caused by microorganisms including bacteria, fungi and microflora (Bell *et al.*, 1996). For a detailed explanation of bone degradation see chapter 5. Yoshino *et al.*, (1991), analysed changes in bone microstructure in three depositional environments. With exposure to air, changes to bone were documented after 15 years and buried bones after five years. Conversely, Bell *et al.*, (1996), documented changes to bone within three months. However, this sample was exposed to scavenging which may have compromised its structural integrity, thus, this result could be regarded as insignificant. Bone weathering has also been suggested as a method for determining the PMI (Buchan and Anderson, 2001, Janjua and Rogers, 2008). Janjua and Rogers (2008), conclude that larger bones such as the femur exhibit more changes than small bones and may provide a better indicator of time since death. This statement seems somewhat obvious as large bones will have a larger surface for degradation processes to manifest. These results show promise but more research is needed to correlate changes to bone microstructure and weathering with time if it is to be successfully applied in a forensic context. Due to a lack of research, such analysis warrants significant experience on behalf of the investigator.

1.9.12 Analysis of soil solutions and detection of biomarkers

Vass *et al.*, (1992), demonstrated how PMI could be established based on biochemicals evolved from the decaying body, in particular microbially produced volatile fatty acids (VFAs) and ions such as sodium (Na^+), chloride (Cl^-), ammonium (NH_4^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), and, sulphate (SO_4^{2}). The experiments were conducted on seven, human, individuals from mixed ancestral backgrounds. During the summer, readings were taken every three days and during the winter every week. Analysis was conducted on the solution from soil underneath the decomposing specimens. The team collected data which may prove useful for determining PMI, namely that propionic, iso-butyric, n-butyric, iso-valeric, and n-valeric acids form in soil in specific ratios during the decomposition process. The team also found that concentrations of Na^+, Cl^-, NH_4^+, K^+, Ca^{2+}, Mg^{2+} and SO_4^{2-} may be useful for determining PMI of skeletonised individuals (Vass *et al.*, 1992). Unfortunately, this

study has several limitations specifically that the sample size was only seven and that the data collected was only relevant to the region where this experiment was conducted (Tennessee, USA). A further, major, limitation of the (presented) research lies within the sampling strategy. Different sampling areas were chosen, with no area being sampled more than once, and Vass *et al.,* (1992) do not seem to have considered the possibility of variation in decomposition products for different areas of the body. Furthermore, when collecting the samples, the bodies had to be tilted. This was unavoidable but may have resulted in mixing the putrescent mass within the corpse itself. It was stated that the methods of analysis are limited when dealing with heat modified bodies or in cases involving mummification (Vass *et al.,* 1992). However, a framework has been produced to allow for others to use the method and collect data relevant to their own environments. But, this is itself a limitation as few other research facilities conduct taphonomic experiments on human remains.

Vass *et al.,* (2002), explored the use of biochemistry in the determination of a PMI estimate using time dependent biomarkers (Vass *et al.,* 2002). This experiment, in contrast to his 1992 work, had a larger sample size. The study was conducted on eighteen individuals over a four year time period. The biomarkers of interest were amino acids, neurotransmitters, and decompositional by-products from various organs such as the liver, kidney, heart, brain and muscle (Vass *et al.,* 2002). Analysis of these markers proved useful and provided a pattern which the team state can be used to estimate a PMI.

Interestingly, the use of volatile fatty acids (VFAs) in forensic cases where the PMI was unknown has proven to be successful. Consequently, these methods exhibit great promise, but further work investigating the effect of clothing, burial, geographical location and precipitation need to be examined further (Buchan and Anderson, 2001).

Statheropoulos *et al.,* (2004), conducted studies of volatile organic compounds (VOCs) produced from a body during decomposition. During the decompositional process volatile organic compounds are produced as intermediate products of decomposition (Statheropoulos *et al.,* 2004). The publication identifies over 80 VOCs including, dimethyl disulphide, toluene, hexane, benzene, 1,2,4-trimethyl, 2-propanone and 3-pentanone. This publication is one of the first to systematically analyse VOCs during putrefaction. It is stated by the authors that VOC analysis could be used forensically to determine PMI but further research and scrutiny are needed along with a much larger sample size.

1.9.13 Assessment of scavenging

Human remains are sometimes modified by animal activity, with some of the most prevalent agents being canids such as domestic dogs and coyotes (Willey and Synder, 1989). It has been suggested that the assessment of scavenging of remains can help in determining the PMI (Buchan and Anderson, 2001). Studies conducted by Haglund (1997) conclude, that canid scavenging results in a fully skeletonised body with the upper limbs missing in as little as 28 days in the Pacific Northwest. Furthermore, disarticulation of all bones except segments of the vertebral column may be expected within two months. After one year, dispersal of the remains may be extensive with many bones not recovered (Haglund, 1997). There are many limitations to using such methods for determining the PMI, including, the number of animals involved with the scavenging (more animals will result in faster disarticulation) (Willey and Synder, 1989), detailed knowledge of the specific species involved (this is not always known), freshness of the corpse in terms of its appeal to scavengers, position and covering of the remains (Haglund, 1997) and human population density (areas of higher population may have fewer scavenged remains) (Haglund *et al.,* 1989, Haglund, 1991). For more general information on the assessment of scavenging in forensic contexts refer to O'Brien *et al*, (2010) and Moraitis and Spiliopoulou (2010).

1.9.14 Assessment of associated death scene material

Often materials are found in association with human remains (tools, weapons, plastics, clothing and paper (Janaway, 2008)). The analysis of their level of degradation may facilitate in determining a PMI (Buchan and Anderson, 2001). A detailed description of the degradation of associated artefacts is presented by Janaway (1996, 2008), and as with human remains, the degradation of associated artefacts is dependent on environmental conditions.
Morse (1983) presents timings for degradation of numerous materials in different environmental conditions. However, such research is scarce but warrants further investigation with more comprehensive and exhaustive studies.

It can be surmised that the processes of decomposition are complex, and the variables that influence them are incalculable. In some instances a body will decompose quickly, and, in others a body can be preserved after thousands of years. It has been established that the PMI

for any death investigation is a paramount question which will ultimately shape the following events, such as, determining if the remains are of forensic importance or identifying the deceased (Forbes and Nugent, 2009).

Although for remains in the early postmortem period there are suitable and accurate methods for determining the PMI, as the postmortem period increases available methods lose accuracy. Furthermore, methods for the later postmortem period are lacking (Forbes and Nugent, 2009) and a dearth of methods is available for buried bodies. This thesis aims to present new methodologies for determining the postmortem interval of buried remains in a forensic context.

Chapter 2: Experimental locations and Soil Analysis

'Who saw him die?'
'I', said the fly
'with my little eye,
I saw him die'
Anon, Who Killed Cock Robin

2.1 Experimental sites for deposition of samples

The experimental depositions occurred at two locations, both part of the University of Wolverhampton. Burials occurring directly in ground were conducted at the Crop Technology Unit, Compton campus, (grid reference SO 88827 98881) (Figure 2.1). Interments occurring in terracotta pots occurred on the roof of the science department, City campus (grid reference SO 91427 98882) (Figure 2.2).

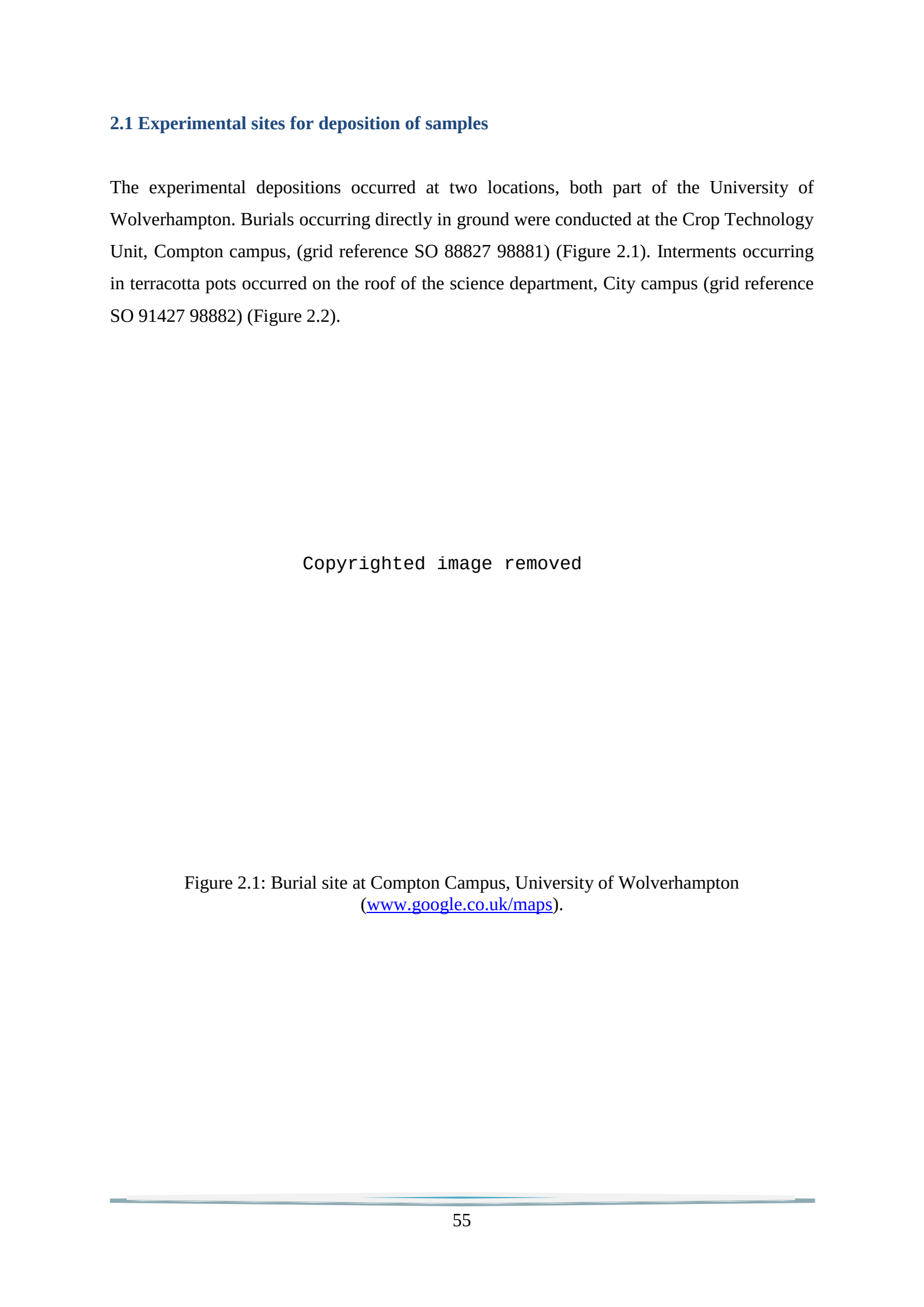


Figure 2.1: Burial site at Compton Campus, University of Wolverhampton (www.google.co.uk/maps).

Figure 2.2: Location of the interment site at City Campus (white box), University of Wolverhampton (www.google.co.uk/maps).

Compton campus is situated in a rural location. The immediate area comprises a mix of localised environments such as forested areas and open ground. In an area of open short grass a pen was constructed of wood and chicken wire to ward of scavenger activity. The chicken wire was interred 60 cm below the ground to prevent scavenger access by digging. The pen was bordered on two of its sides by lines of trees.

At City campus, terracotta pots containing Compton soil were used to inter samples on the roof. The roof houses two sheds and two green houses. The pots were positioned against a brick wall but exposed to both sun and shade as the day progressed.

The soil used in this research is a sandy silt loam as determined by Vaz (2001).

2.2 Methods for soil analysis

2.2.1 Determination of soil pH

In the first instance Compton soil was sieved to < 2 mm. Ten grams of the sieved product was placed into a beaker along with 25 ml of distilled water. The solution was mixed for 15 minutes on a magnetic stirrer (Rowell, 1994). A pH probe (VWR International, pH100) was calibrated using pH 4, pH 7 and pH 9 buffers and utilised to record the soil pH. The electrodes were placed directly into the solution avoiding contact with the side and bottom of the beaker. A record of the pH was taken when the value had stabilised.

2.2.2 X-Ray Fluorescence (XRF) of Compton soil

X-ray fluorescence (XRF) allows for the identification of an element by measuring its characteristic X-ray wavelength (Jenkins, 1988, p.76). The sample is bombarded with X-rays and the electrons undergo excitation and relaxation whereby they jump between the different shells of an atom. The energy emitted via this process is characteristic for the atomic number of the emitting element (Muller, 1972). Quantification of an element is determined by measuring the emitted line intensity and then relating this to the elemental concentration (Jenkins, 1988, p.75). For further information on XRF please refer to Christian and O'Reilly (1986 p.412) and, Higson (2003, p.158).

Compton soil was dried in an oven overnight (24 h) at 40°C after which the dried soil was ground and sieved to 100 µm. 8.5 g of this product was combined with 1.5 g licowax to aid binding. The mixture was homogenised and pressed into a disc under a pressure of 12 tonnes using a pellet press. The disc was placed into the XRF (Xepos, Spectro) sample holder ready for analysis.

2.3 Results

2.3.1 pH of Compton Soil

The pH of Compton soil was found to be acidic with an average of 5.54.

Reading 1: 5.72

Reading 2: 5.26

Reading 3: 5.64

Average = 5.54

2.3.2 XRF results from Compton Soil

Determination of elemental composition by XRF analysis (Figure 2.3) concluded that the soil is made up of numerous elements with silicon, aluminium, iron, and potassium being the most concentrated. The data is presented as a concentration in parts per million. The order of the elements presented reflects the order of the results generated during analysis. No elements were omitted from the results.

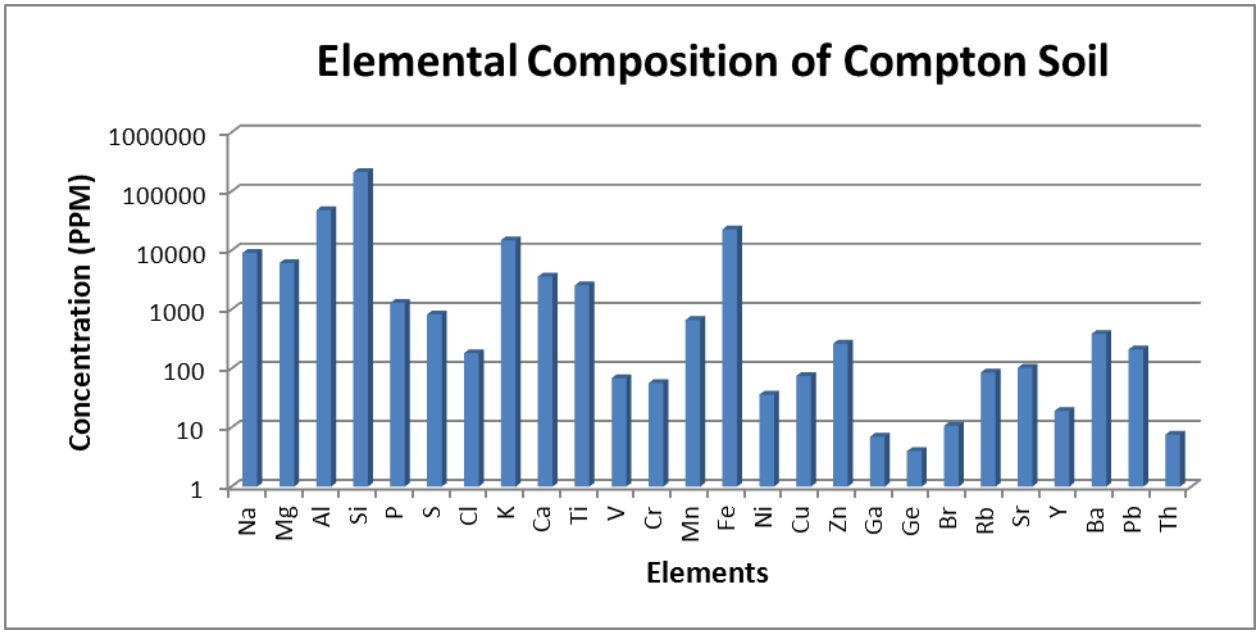


Figure 2.3 : The major elemental composition of Compton Campus soil.

2.4 Discussion

Two locations but one soil type was used for the interment of the samples. Soil analysis illustrated that the soil is acidic and comprised of numerous elements with silicon, aluminium and iron being most prevalent. A previous study at Compton campus (Vaz, 2001) stated that Compton soil is a sandy silt loam. Sandy soils have a certain degree of water permeability as opposed to clay soils (Bethell and Carver, 1987) thus may not retard the decomposition process due to minimal waterlogging. At present little is known about the influence of the soil pH on the decomposition process but it may be inferred that acidic soils may slow down the decomposition process by inhibiting bacterial growth (Tibbett and Carter , 2008). Little is known about the effects of specific chemical elements on the decomposition process but it is stated that metal ions in a burial environment provide localised toxicity which may retard microbial activity (Janaway, 1996). Furthermore, it is suggested that inorganic elements such as phosphorus, sulphur, calcium and potassium rarely retard microbial activity (Hopkins, 2008). Soil analysis was conducted to further understand the environment used in this research, but more research is needed to correlate specific characteristics and changes in the geochemistry with the decomposition processes. This data has highlighted the need for multidisciplinary approaches in forensic taphonomic contexts.

Chapter 3: Cartilage Decomposition

She is cold :
Her blood is settled, and her joints are stiff ;
Life and these lips have long been separated :
Death lies on her like an untimely frost
Upon the sweetest flower of all the field.

Capulet, Romeo and Juliet
Act IV, Scene V

3.1 Cartilage Structure

During the fifth week of embryonic development cartilage develops from mesenchymal cells that aggregate to form a blastema (Bhosale and Richardson, 2008). These undifferentiated mesenchymal cells cluster together and form collagens, proteoglycans and non-cartilaginous proteins (Buckwalter and Mankin, 1997). This cluster of cells becomes recognisable as cartilage once a sufficient matrix has developed and pushed cells apart (Buckwalter and Mankin, 1997). During development cartilage is at its highest metabolic rate and cell density is high compared to developed cartilage (Buckwalter and Mankin 1997).

Depending on the composition of its matrix cartilage can be classed as elastic, fibro or hyaline. Cartilage is essentially one type of cell contained within an extra cellular matrix (Buckwalter and Mankin, 1997, Kühn *et al.*, 2004, Thomas *et al.*, 2007). It lacks a blood supply, lymphatic vessels and nerves (Buckwalter and Mankin, 1997, Bhosale and Richardson, 2008, Hendren and Beeson, 2009). Articular cartilage covers articular surfaces of synovial joints (Kerin *et al.*, 2003) allowing for friction free movement between bones (see figure 3.1), it is specialised and absorbs impact resultant from movement and consequently protecting the underlying bone (Yeh *et al.*, 2005).

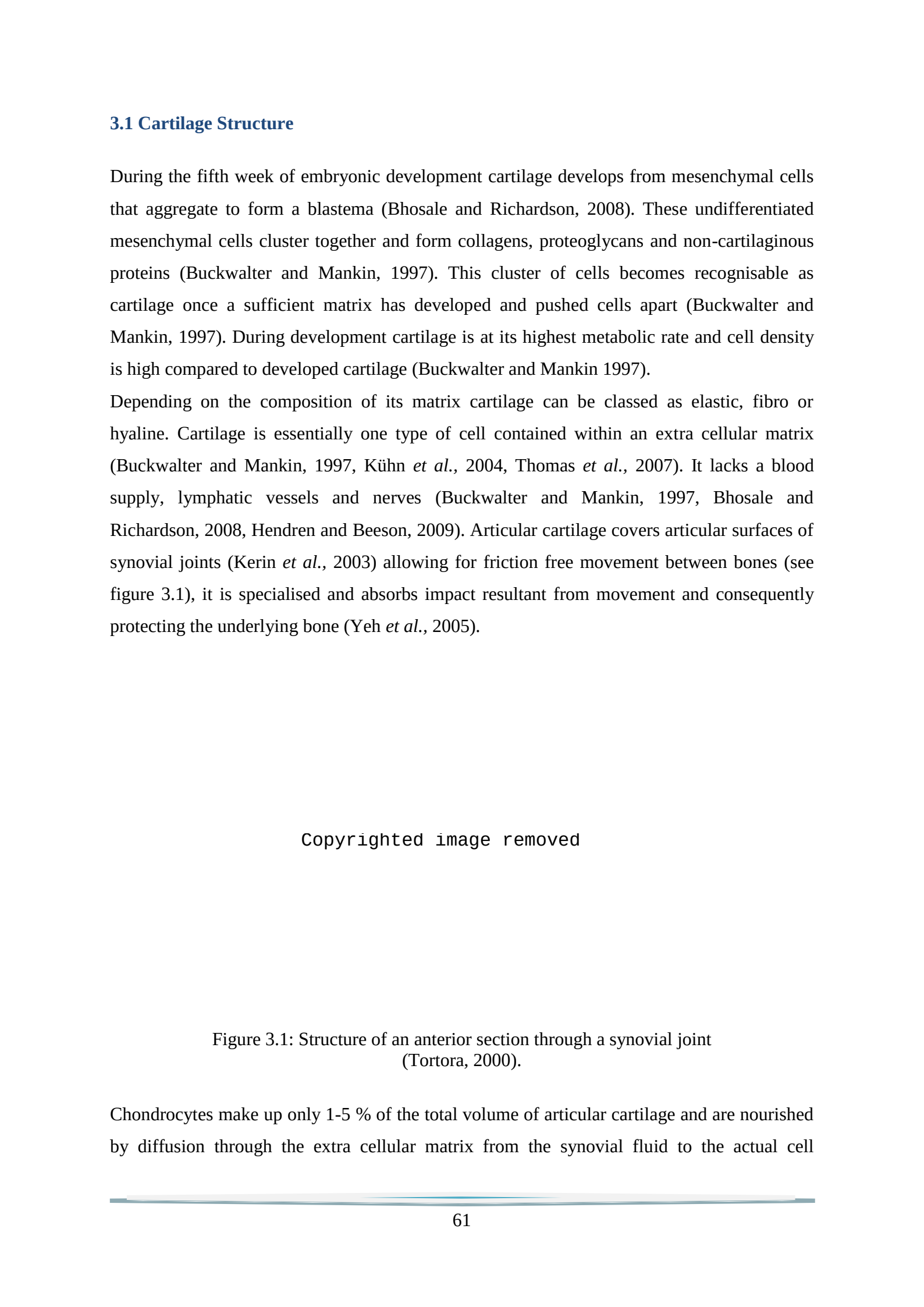


Figure 3.1: Structure of an anterior section through a synovial joint (Tortora, 2000).

Chondrocytes make up only 1-5 % of the total volume of articular cartilage and are nourished by diffusion through the extra cellular matrix from the synovial fluid to the actual cell

(Bhosale and Richardson, 2008, Hendren and Beeson, 2009) consequently depending on anaerobic metabolism (Drobnič, 2005). They contain numerous organelles including endoplasmic reticulum, golgi apparatus and in some instances, intracytoplasmic filaments, lipids, glycogen, secretory vesicles and cilia (Buckwalter and Mankin, 1997). Chondrocytes do not form cell to cell contacts but are active, similar to vascular tissue, but due to their low abundance they contribute little to the total metabolic activity (Buckwalter and Mankin, 1997). Cartilage is comprised of water, collagen and proteoglycans. Water makes up 65-80% of the wet weight of cartilage (Bhosale and Richardson, 2008). The proteoglycans provide the compressive strength and support fluid and electrolyte balance of cartilage (Bhosale and Richardson, 2008). The negatively charged proteoglycans are aggregated to a strand of hyaluronic acid and secondarily attached to type II collagen (Yeh *et al.,* 2005, Bhosale and Richardson, 2008).

Overall, cartilage structure is reported to vary between species and anatomical locations. Hendren and Beeson (2009), use human cartilage from the knee and ankle as an example.

The surface of cartilage in its unworn state appears smooth and lacks any surface morphological features (Graindorge and Stachowiak, 2000). However, this is dependent on the method of sample preparation and the use of an environmental scanning electron microscope (ESEM) over a scanning electron microscope (SEM) (Graindorge and Stachowiak, 2000) as ESEM allows for the analysis of wet, non-conductive samples. Rolauffs *et al.,* (2008) report that chondrocytes are arranged in distinct patterns if visualised from the superior aspect, parallel to the articular surface. They state that chondrocytes can be arranged in strings, clusters, pairs and singularly (Rolauffs *et al.,* 2008). Articular cartilage is made up of four zones; the superficial, transitional / middle, deep, and, calcified zone (Buckwalter and Mankin, 1997, Yeh *et al.,* 2005, Bhosale and Richardson, 2008) as illustrated in figure 3.2.

Figure 3.2: Illustrates the zones that make up articular cartilage. Taken from Yeh et al., (2005).

The superficial zone typically consists of two layers and contains ellipsoid shaped chondrocytes aligned so their axes are parallel to the articular surface (Buckwalter and Mankin, 1997). The cells are covered in a synovial fluid also referred to as the lamina splendens or lubricin (Bhosale and Richardson, 2008). This zone contains high water content and acts as a filter for large molecules (Bhosale and Richardson, 2008). The transitional or middle zone houses spheroid chondrocytes and the overall cell density is lower. The cells contained in the deep or radial zone are spheroidal but arranged in columns perpendicular to the cartilage surface (Buckwalter and Mankin, 1997). The calcified zone contains cells embedded in a calcified matrix and exhibits a lower metabolic activity (Bhosale and Richardson, 2008). Reinert *et al.,* (2002) lists the elemental composition of cartilage, namely the presence of phosphorus (P), sulphur (S), potassium (K), chlorine (Cl), calcium (Ca) and the matrix elements, hydrogen (H), carbon (C), nitrogen (N) and oxygen (O). Cartilage cells must work in equilibrium to maintain the cartilage structure. A deviation in this equilibrium may result in cell death, a change in architecture or change in composition which may result in a pathology such as osteoarthritis (Yeh *et al.,* 2005).

Very little is known about the processes occurring to cartilage postmortem and such phenomena have received little attention. It is stated that chondrocytes are still viable after death (Drobnič *et al.,* 2005) and may be less susceptible to degrading microorganisms due to the avascular qualities of the tissue (Gino *et al.,* 2003). A study conducted by Lasczkowski *et al.,* (2002) visualised postmortem chondrocyte loss by vital staining and confocal laser scanning 3D microscopy. In their study, they concluded that loss of chondrocytes was time and temperature dependent.

3.2 Methods

Due to the similarities between porcine and human samples in aspects of body composition (Book and Bustad, 1974, Brambilla and Cantafora, 2004), porcine hind trotters were used as human analogues for this research. Porcine substitutes are common in forensic studies into decomposition processes (Morten and Lord, 2002) and are classified as an adequate comparative model (Weitzel, 2005).

Porcine hind trotters were interred in soil to a depth of 15-20 cm at the University of Wolverhampton Crop Technology Unit, Compton Campus. The soil type at this site is sandy silt loam (Vaz, 2001) with an average pH of 5.5.

A total of eight replicates took place during a three year period. Four replicates took place during winter to spring and a further four taking place in spring to summer. Two trotters were placed directly into each grave. To facilitate easy excavation, the trotters were attached to long pieces of bright coloured garden string or, the four corners of the graves were marked with sticks, allowing for easily identifiable samples even in long overgrown vegetation. Excavation occurred at various intervals until skeletonisation was observed. In the first instance excavations occurred weekly but later modified to shorter intervals, e.g. four, 11, 14, 18, 22 days (see Table 3.1 for disinterment intervals). The initial weekly excavations allowed for the assessment of time taken for skeletonisation to occur.

During sample excavation soil was removed in small layers (Hunter and Dockrill, 1996) until the trotters were exposed. This allowed for careful excavation minimising postmortem damage to the sample which may have caused confusion when later analysis took place. The samples were lifted (sometimes block lifted in soil to prevent damage to the sample), placed in sealed plastic containers and stored at -20˚C to prevent further degradation.

Table 3.1: Disinterment intervals for porcine trotters during the three year period. Spring to summer and autumn to winter are shown. Note, D refers to days.

Spring - Summer							
Burial Interval (Days: D)							
Replicate 1		Replicate 2		Replicate 3		Replicate 4	
Interval	Date	Interval	Date	Interval	Date	Interval	Date
7 D	01.04.08	7 D	25.03.09	4 D	10.04.09	7 D	15.07.09
14 D	08.04.08	49 D	13.05.09	11 D	17.04.09	13 D	21.07.09
21 D	15.04.08	70 D	03.06.09	14 D	20.04.09	15 D	23.07.09
28 D	22.04.08	91 D	24.06.09	18 D	24.04.09	20 D	28.07.09
35 D	29.04.08			22 D	28.04.09	23 D	31.07.09
42 D	06.05.08			25 D	01.05.09	27 D	04.08.09
49 D	13.05.08			28 D	04.05.09	30 D	07.08.09
56 D	20.05.08			32 D	08.05.09	33 D	10.08.09
63 D	27.05.08			35 D	11.05.09	37 D	14.08.09
70 D	03.06.08			42 D	18.05.09	45 D	21.08.09
77 D	10.06.08					48 D	25.08.09
84 D	17.06.08						
91 D	24.06.08						
98 D	01.07.08						
105 D	08.07.08						
Autumn - Winter							
Burial Interval (Days: D)							
Replicate 5		Replicate 6		Replicate 7		Replicate 8	
Interval	Date	Interval	Date	Interval	Date	Interval	Date
7 D	02.10.08	21 D	21.09.09	3 D	05.11.09	2 D	02.02.10
14 D	09.10.08	42 D	12.10.09	6 D	08.11.09	5 D	05.02.10
21 D	16.10.08	63 D	02.11.09	9D	11.11.09	8 D	08.02.10
28 D	23.10.08	84 D	23.11.09	12 D	14.11.09	10 D	10.02.10
35 D	30.10.08	105 D	14.12.09	15 D	17.11.09	13 D	13.02.10
42 D	06.11.08			17 D	19.11.09	16 D	16.02.10
49 D	13.11.08			20 D	22.11.09	19 D	19.02.10
56 D	20.11.08			23 D	25.11.09	22 D	22.02.10
63 D	27.11.08			26 D	28.11.09	24 D	24.02.10
70 D	04.12.08			29 D	01.12.09	26 D	26.02.10
77 D	11.12.08			32 D	04.12.09	30 D	02.03.10
84 D	18.12.08			35 D	07.12.09	33 D	05.03.10
91 D	23.12.08			39 D	11.12.09	36 D	08.03.10
98 D	02.01.09			42 D	14.12.09	40 D	12.03.10
105 D	09.01.09					44 D	16.03.10
						46 D	18.03.10

3.2.1 Burial set up

Figure 3.3 details the typical sample layout at the Compton campus facility. Two porcine trotters (figure 3.3A) were interred into each grave (figure 3.3B). All the samples were covered with soil and grass, and the graves were clearly marked to allow for easy identification in long vegetation. Long and dense vegetation was problematic in the spring and summer months.

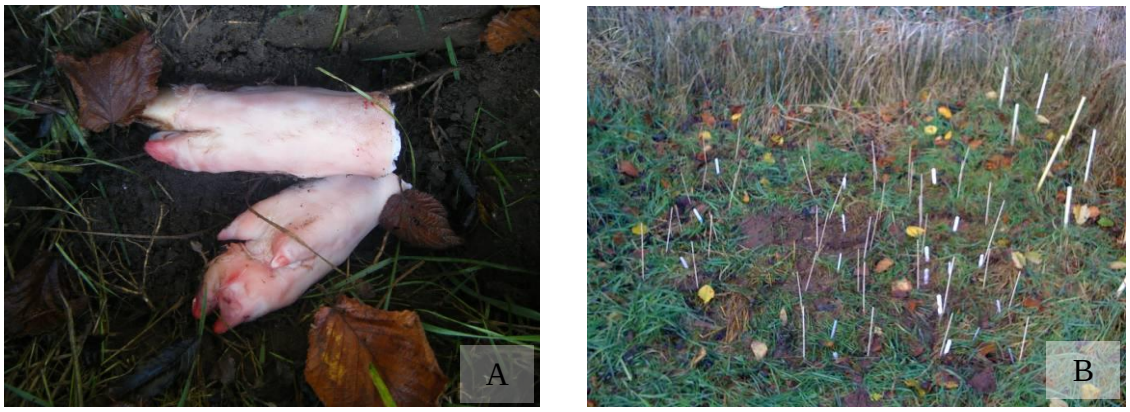


Figure 3.3: A typical sample layout, graves were to a depth of 15 centimetres. The four corners of the grave were marked with wooden stakes. A illustrates an individual burial and B shows the site after the samples had been buried.

3.2.2 Macroscopic analysis and dissection of porcine trotters

The entire porcine trotter was subjected to macroscopic analysis to record any visually discernible changes in overall appearance. Photographic evidence was obtained using a digital camera (Coolpix 4500, Nikon). Recorded changes to the trotters included decompositional changes to the dermis, musculature and cartilage.

Superfluous soil adhering to the trotters was removed, where possible, to obtain clearer images. This was conducted by gentle peeling and removal of soil using a blunt spatula and a brush. However, if fungal structures, or skin slippage were evident cleaning was kept minimal until completion of documentation. Trotters were then dissected using the presented method (section 3.2.3) and photodocumentation of exposed cartilage was undertaken. Analysis of the cartilage at the macroscopic level allowed for the application of a grading system as first proposed by Mcllwraith *et al.*, (1987) and later modified by Fuller *et al.*, (2001). This grading system allowed for the documentation of the colour, the amount of coverage and the texture of the cartilage *in situ*.

3.2.3 Dissection method for porcine trotters

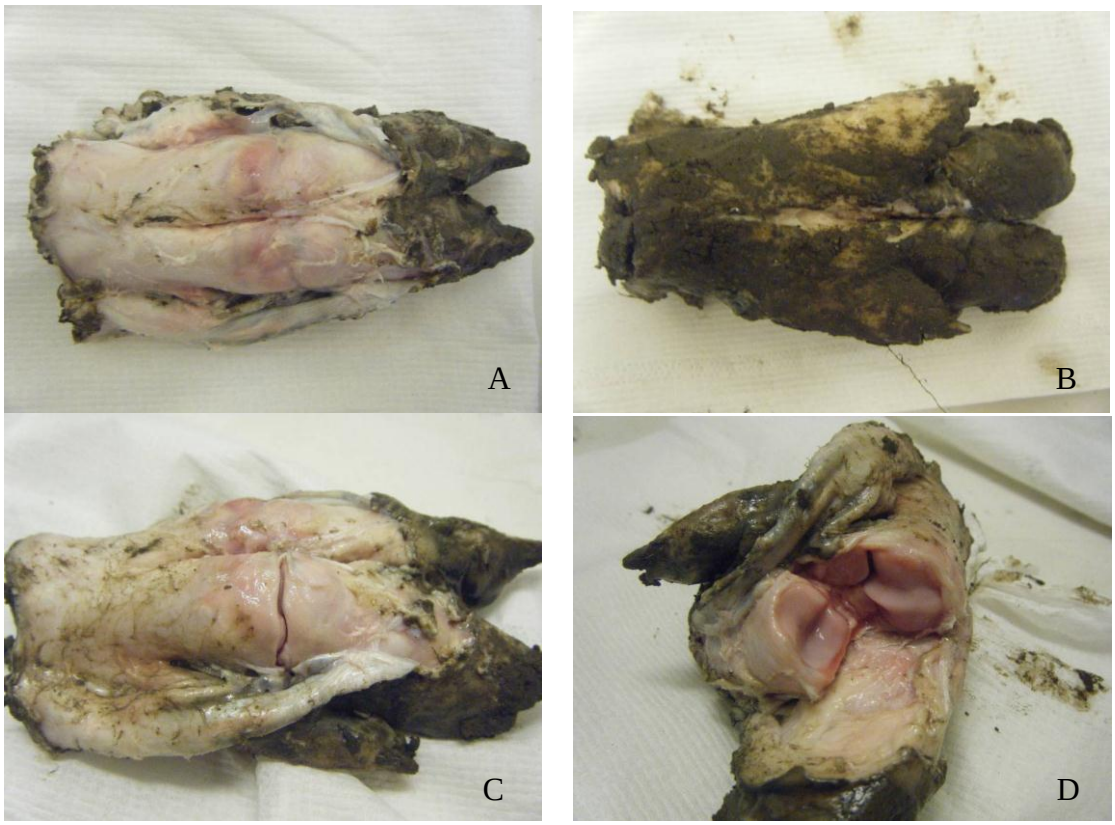


Figure 3.4: The dissection method developed for porcine samples.

Figure 3.4 shows the dissection method used on the porcine samples. Incisions were made using a sterile scalpel. A new blade was utilised for each dissection. The dermis on the superior aspect of the trotter was incised with a cut progressing down the middle of the sample in between the metatarsals and phalanges. The dermis was carefully cut and peeled back exposing the underlying tissue (figure 3.4A). The trotter was inversed and a similar incision was made on the inferior aspect (figure 3.4B). The synovial membrane was cut exposing the joint space (figure 3.4C). Synovial fluid was collected using a sterile syringe and needle. Any ligaments and tendons were dissected allowing for free manipulation of the desired joint and consequent photodocumentation of the cartilage surface and samples of cartilage to be acquired (figure 3.4D). The scalpel blade was sterilised with ethanol and passed through the flame of a Bunsen burner after each stage of the dissection, thus minimising the risk of contamination of the cartilage surface with soil debris.

Exposed cartilage was dissected using a scalpel with a gentle sawing action and approximately 1cm x 1cm samples were obtained. These were placed into a labelled Petri dish

and frozen at -20˚C until further analysis. Cartilage was dissected carefully to ensure the articular surface was identifiable.

3.2.4 Cartilage sectioning

Extracted cartilage was embedded in a 70% gelatine solution, frozen in liquid nitrogen and sectioned to a thickness of 7-12 μm. Samples were sectioned using a cryostat (Cryocut-E, Reichert-Jung) at a temperature of -20°C. This was to ensure that the samples did not degrade further due to an elevated temperature. The sections were transferred with dissection tweezers to a standard microscope slide for histological staining.

3.2.5 Cartilage staining

Two histological stains were used for staining the cartilage sections. Haematoxylin and Eosin (H&E), was applied to the sectioned samples. H&E recipe consisted of a) Haematoxylin (Haematoxylin 5 g, Ethyl alcohol 50 ml, potassium alum 100 g, distilled water 950 ml, mercuric oxide 2.5 g and glacial acetic acid 40 ml) and b) Eosin (1 crystal of phenol was added to prepared solution). H&E stains cell nuclei dark pink to blue (Bancroft and Cook 1984, p.21). Haematoxylin was applied to the tissue section and left for two minutes. The excess stain was washed off using tap water. Eosin was then applied to the section and left for 30 s. Finally, the section was washed with distilled water.

Similarly, Titan yellow (premade) was utilised to test for the presence of magnesium within the cartilage sections. Three drops of 0.2% aqueous titan yellow and three drops of 2-N-NaOH were applied to the tissue section and left for 45 minutes. Then, the titan yellow was washed off using distilled water.

After, both the titan yellow and H&E stained sections were fixed in polystyrene and xylene (DPX) and covered with a standard microscope cover slip. The slides were transferred in to a slide holder to prevent damage and stored in a dark cupboard.

3.2.6 Light microscopy of stained tissue sections

Light microscopy analysis was conducted on the prepared slides using an Eclipse E600 (Nikon) with a camera attachment. Magnification was at 400x and resulting images were not digitally modified.

3.2.7 Scanning electron microscopy / Energy dispersive X-ray analysis (SEM-EDX) of porcine cartilage

SEM analysis of dissected cartilage was conducted with an EVO 50 scanning electron microscope (Zeiss) in the cool stage (Deben). This ensured that the samples did not thaw during analysis which might have altered the tissue structure. The samples were mounted on carbon stubs with the articular / synovial surface facing up. Scanning electron microscopy coupled with energy dispersive X-ray (SEM-EDX) (EDX: Oxford Instruments, Inca X-Sight) was used to analyse the chemical composition of surface crystals. Further analysis implemented the use of the EDX mapping feature which allowed for the position of individual elements to be mapped on the analysed sample.

3.2.8 Culturing of bacteria from cartilage

A specialist medium (B-41) rich in magnesium was utilised to culture bacteria contained on the cartilage surface. Sections of cartilage and small amounts of synovial fluid were inoculated on the B-41 medium and incubated at 30˚C until the formation of crystals was observed. Bacterial subculturing was conducted to ensure pure colonies of bacteria were obtained ready for identification. 200 ml of B-41 medium: (wt/vol) 0.2% $MgSO_4$. H_2O, 0.2% K_2HPO_4, 0.4% yeast extract and 1.6% Bacto Agar (Difco). The pH was adjusted to 7.5 with 0.1 M NaOH (Rivadeneyra *et al.*, 1992).

3.2.9 Analysis of pH at the cartilage synovial surface

The pH of the cartilage synovial surface was tested using universal indicator strips pH 1-11 (Johnson test papers). The strip was placed over the cartilage surface and the result compared to the manufacturers charts and verified by three different analysts.

3.2.10 X-ray diffraction (XRD) analysis of crystals located on the cartilage surface

Crystalline structures are comprised of an internal structure arranged in a precise and regular way which is periodic within the crystal anatomy (Pickworth Glusker and Trueblood, 1972). X-ray diffraction (XRD) analysis can be used to identify crystalline structures. X-rays are bombarded on to the sample and upon hitting the crystalline structure are diffracted away and detected by a diffractometer. This allows for qualitative identification based on measuring the angles of diffraction (Christian and O'Reilly, 1986, p.414). The data collected using XRD results in a relative intensity (I) for each reflection, and its value for the scattering angle (2 theta) (Pickworth Glusker and Trueblood, 1972).

Crystals located on the cartilage surface were analysed in two formats; 1) cartilage housing crystals was adhered to a sticky pad and placed into the XRD and analysed and 2) Crystals precipitated by bacteria on the B-41 medium were collected by scraping a sterile scalpel blade across the surface of the media. These crystals were fixed to an adhesive pad and attached to a specialised glass slip ready for insertion in to the XRD (PW 1729 X-ray generator, Philips).

3.2.11 Extraction of bacterial fatty acids from bacteria found on cartilage

Extracted bacterial fatty acids can be analysed using gas chromatography – mass spectroscopy (GC-MS) to aid in identification of the bacterial species. Fatty acids, from bacterial species cultured from cartilage, were extracted using the method presented by Sasser (1990) who explains that short chain fatty acids have been routinely used to identify anaerobic bacteria. The methodology cleaves fatty acids from lipids and the data generated from such analysis is compiled within a database allowing for possible identification if a match is generated.

Four reagents were prepared, reagent one for saponification (45 g sodium hydroxide, 150 ml methanol, 150 ml distilled water), reagent two for methylation (325 ml of 6.0N hydrochloric acid, 275 ml methyl alcohol), reagent three for extraction (200 ml hexane, 200 ml methyl tert-butyl ether), and, reagent four for clean-up of the sample (10.8 g sodium hydroxide, 900 ml distilled water).

Bacteria were harvested from the purified cultures and inoculated on Trypticase Soy Broth Agar (TSBA) (30 g Trypticase Soy Broth and 15 g of Agar per litre) by quadrant streaking and left to incubate for 24 h at 28°C. Such quadrant streaking allows for standardisation of bacterial physiological age if samples are obtained from the same quadrant in all experiments.

Forty mg of bacteria were harvested from quadrant three and placed into a sterile culture tube using a 4 mm loop.

1.0 ml of reagent one was added to each culture tube and vortexed for 10 sec. The tubes were placed into a boiling water bath for 5 min and then vortexed for a further 10 sec and then returned to the boiling water bath for 25 min.

The culture tubes were allowed to cool, after which 2 ml of reagent two was added. The tubes were vortexed for 10 sec and heated at 80°C (+/-1) for 10 min (+/-1). After heating, the culture tubes were rapidly cooled in a freezing water bath.

1.25 ml of reagent three was added and the tubes were tumbled on a clinical rotator for 10 min. Then the aqueous (lower) phase of the solution was pipetted out and discarded.

3 ml of reagent four was added to the remaining solution and tumbled for a further 5 min. Then, 2/3 of the organic (top) phased was transferred into a Gas Chromatography (GC) vial ready for analysis with GC-MS. GC-MS analysis was conducted by Professor Zofia Piotrowska-Seget at the Uniwersytet Slaski, Poland.

3.2.12 Testing for crystal formation on cartilage absent of soil

To test for the formation of crystals outside of a soil based environment trotters were placed into sealed plastic boxes (to prevent mummification and access by insects) and placed on the roof of the City Campus MA block (see figure 2.2). The samples were collected after seven days, dissection and analysis with SEM, SEM-EDX followed using the aforementioned methods (see chapter sections 3.2.3, and 3.2.7).

3.2.13 Percolation of collected rain water through soil

To test the effects of rain water on buried samples rain water was collected after a heavy shower. The pH of the collected water was measured using a pH probe (VWR International, pH100). The probe was calibrated and placed into the rain water and a reading was taken when the result stabilised. The collected rain water was allowed to naturally percolate through Compton soil. The resultant solution was filtered to remove particles. This solution was then placed onto the cartilage surface using a sterile plastic pipette.

3.2.14 Application of cartilage decomposition to other animal species

To determine whether the crystals observed during the decomposition of porcine cartilage formed in other animal species, burials of cow and goat limbs were conducted. The cow and goat samples were collected from a market located in Wolverhampton. The samples were fresh and collected no more than two days after slaughter of the source animal.
The aim of this series of experiments was not to determine when, but if the crystals formed. Burial and analysis followed the same methodologies as described in chapters 3.2.2, 3.2.3 and 3.2.7.

3.2.15 DNA analysis of a crystal producing bacteria found on cartilage

Crystal producing bacteria were inoculated onto B-41 agar slopes and incubated at 30ºC for three days. Two agar slopes were then packaged into a box and sent to the DNA analysis service, NCIMB Ltd. The resulting DNA sequence was analysed using a Basic Local Alignment Search Tool (BLAST) (www.blast.ncbi.nlm.nih.gov/Blast.cgi). This works by analysing the query DNA sequence and matching it against sequences already held within a database (Krane and Raymer, 2003, p.50, Lesk, 2008, p.192). The sequence was input (in FASTA format) into the nucleotide blast function and run against the nucleotide collection held within the database.

3.3 Results

As expected the pig trotters exhibited a gradual degradation over time observed by the presence of intact soft tissues in the first instance (the first few weeks), ending with complete or partial skeletonisation after 13 weeks of burial.

3.3.1 Macroscopic analysis

The macroscopic results are split into two sections, the first details the analysis between 0-32 days postmortem (replicate three) as this is when the decomposition processes had the most effect. The second part will detail the analysis between 0-13 weeks (replicate two).

0-32 days postmortem

Control (0 days burial)

Figure 3.5 illustrates the dissected control trotter (with no exposure to soil) and shows soft tissues in a healthy unremarkable condition. The cartilage was white and glossy in appearance and no evidence of decomposition was seen.

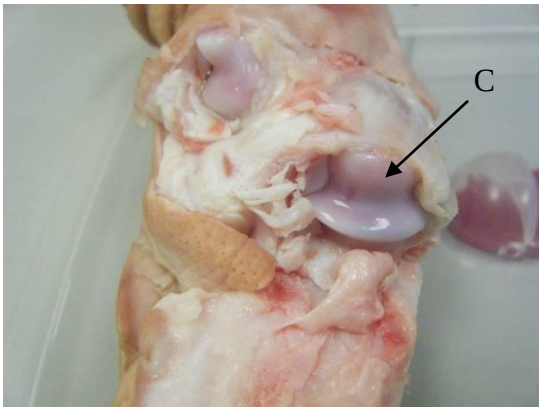


Figure 3.5: Control cartilage (0 days burial). C refers to the cartilage.

4 days post burial

At four days post burial the trotter skin began to discolour possibly due to interaction with the soil matrix. Residual soil was still in association and left *in situ* to prevent damage to the specimen (Figure 3.6A). Figure 3.6B illustrates the dissected view of the four day specimen with the cartilage exposed. It can be seen that the cartilage was white / grey in colour with a glossy appearance, similar to the control.

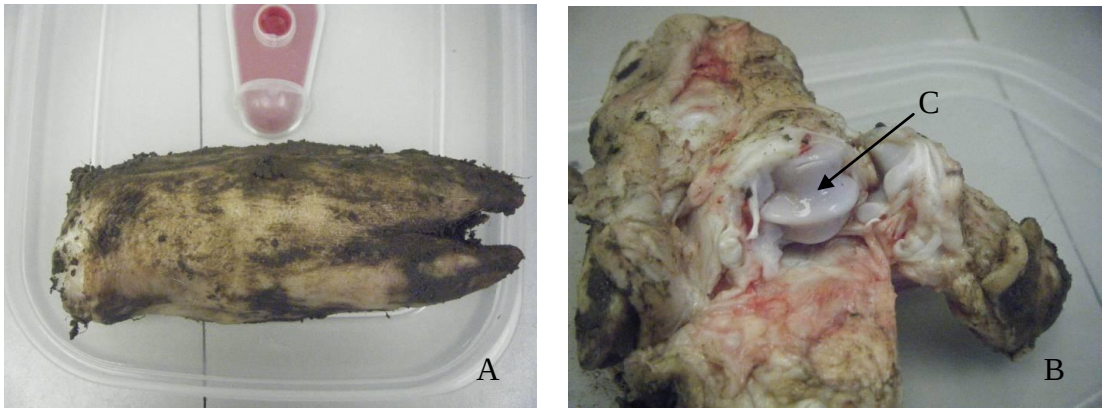


Figure 3.6: Porcine trotters at 4 days post burial. C refers to the cartilage.

11 days post burial

Figure 3.7A illustrates the specimen at 11 days post burial showed a discolouration resulting in a darker appearance than the previous samples. Fungal structures were noted on the dermis (Figure 3.7B). The exposed cartilage was white and glossy in colour (Figure 3.7C).

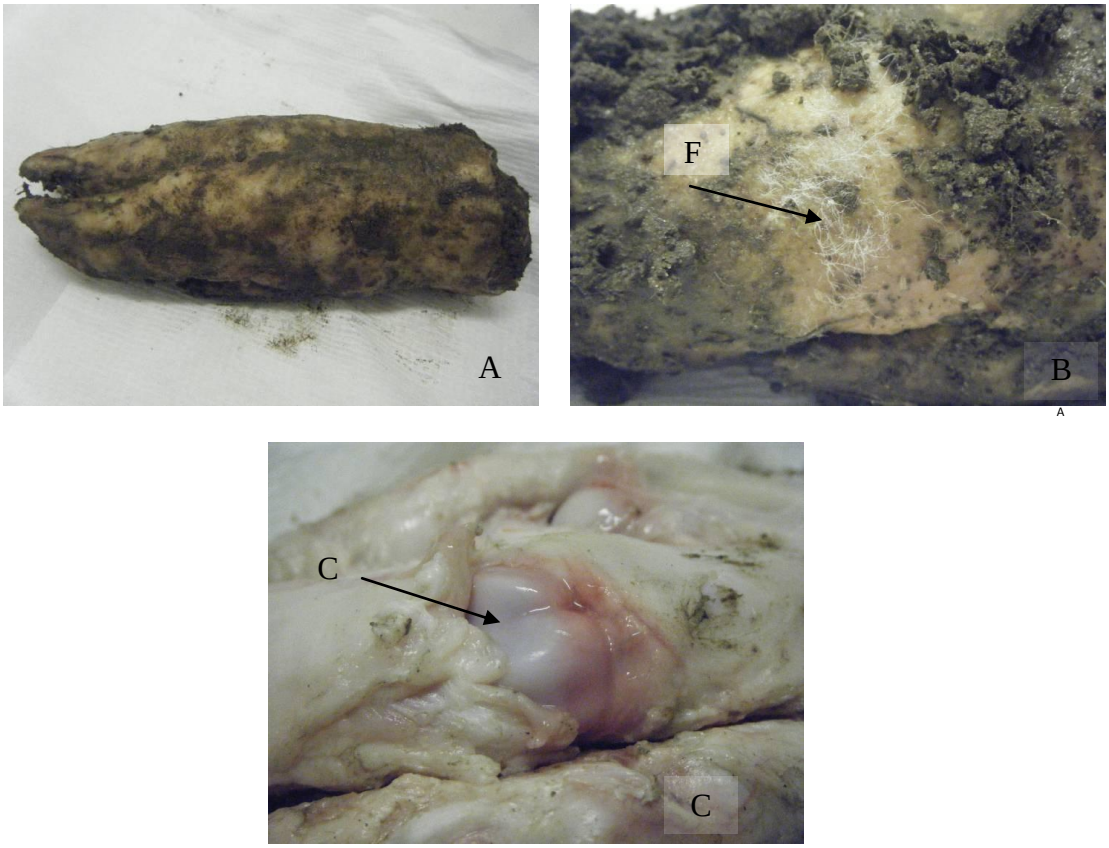


Figure 3.7: Porcine samples at 11 days post burial. C: cartilage, F: fungi.

14 days post burial

Figure 3.8A illustrates the presence of skin slippage on the lateral edge of the trotter at 14 days post burial. The exposed cartilage exhibited a white / pink glossy colouration (figure 3.8B).

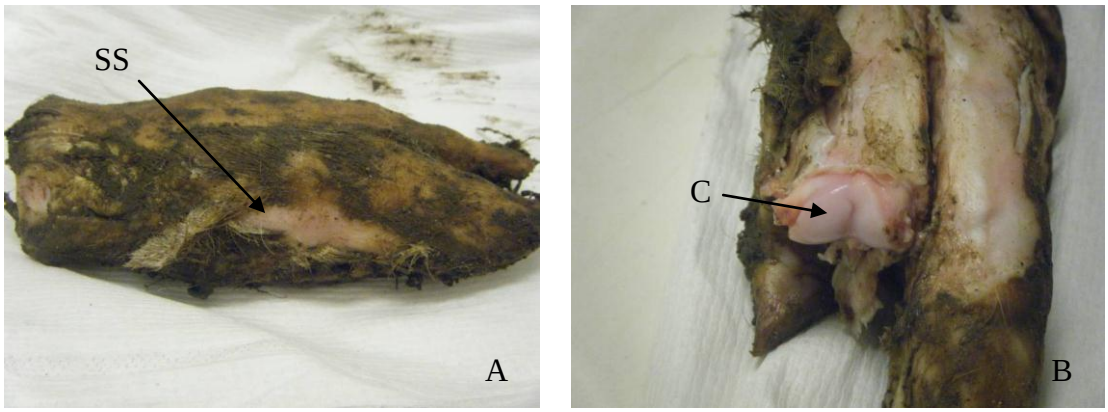


Figure 3.8: Porcine samples at 14 days post burial. C: cartilage, SS: skin slippage.

18 days post burial

At 18 days post burial (figure 3.9A) the dermis of the trotter had darkened with some areas of skin slippage evident. Figure 3.9B illustrates the pink colouration has become darker compared to previous samples.

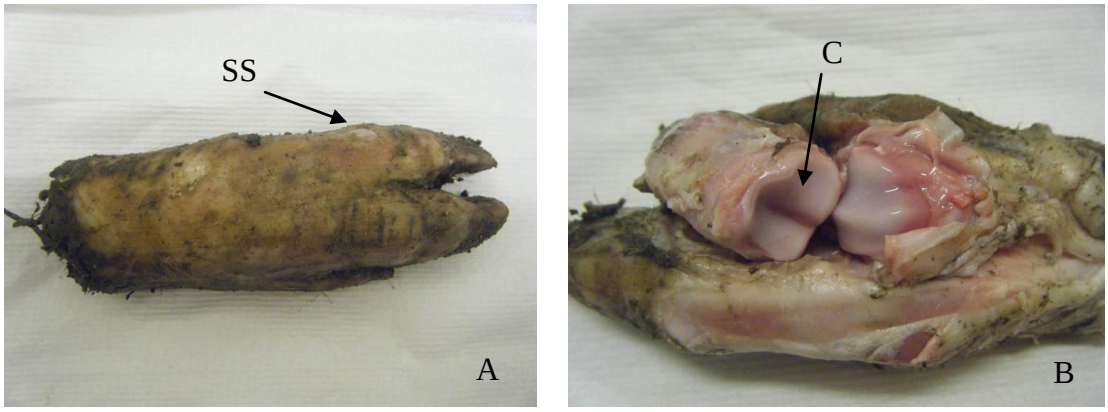


Figure 3.9: Porcine sampled after 18 days burial. C: cartilage, SS: skin slippage.

22 days post burial

Insect activity was prevalent on the 22 day sample (Figure 3.10A). Maggot infestation was pronounced around the amputation site of the trotter (Figure 3.10B). However, the maggot activity had not compromised the joint space as the joint capsule was intact. The cartilage exhibited a strong pink colouration (Figure 3.10C). Note: the areas of damage to the cartilage were a result of sample collection and not postmortem processes.

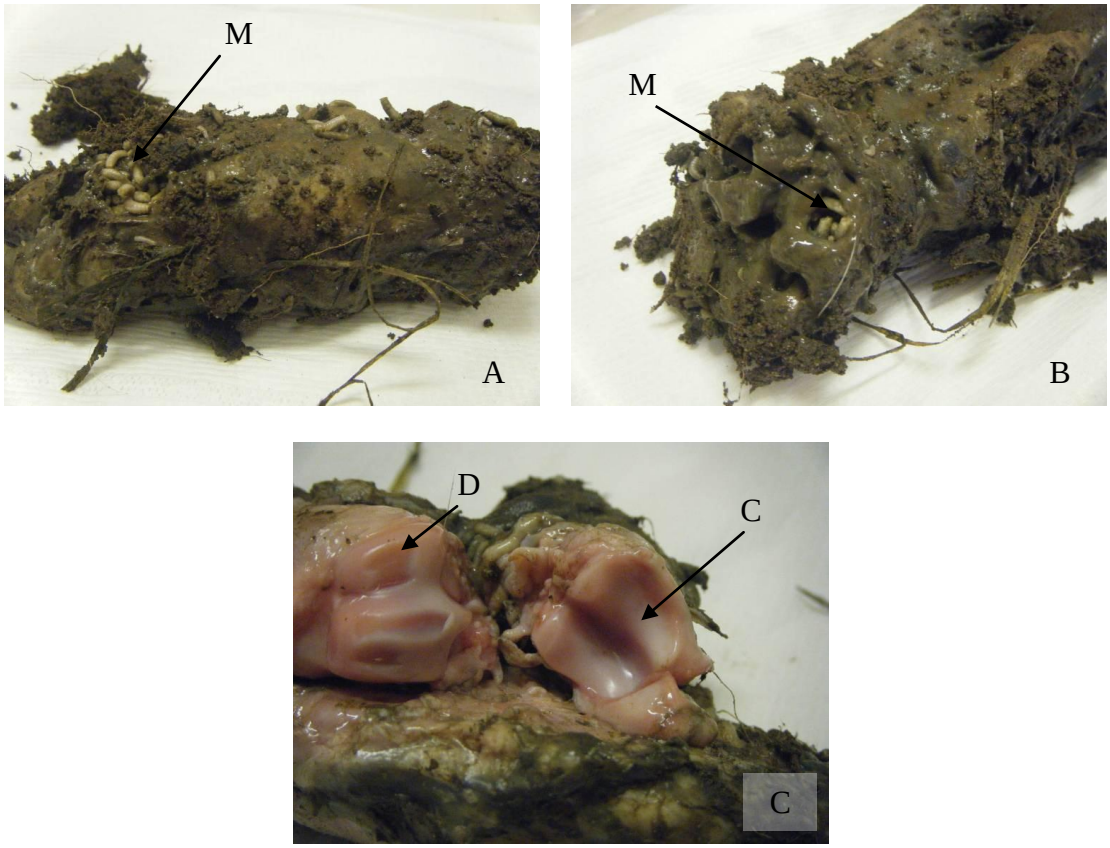


Figure 3.10: Porcine trotters after 22 days of burial. C: cartilage, D: areas of cartilage collection damage, M: maggot activity.

25 post burial

At 25 days post burial skin slippage was pronounced. Therefore cleaning of this sample was kept to a minimum to prevent further damage (Figure 3.11A). As with the previous sample, maggot activity was located at the amputation site (Figure 3.11B). Figure 3.11C illustrates the pink colouration of the cartilage.

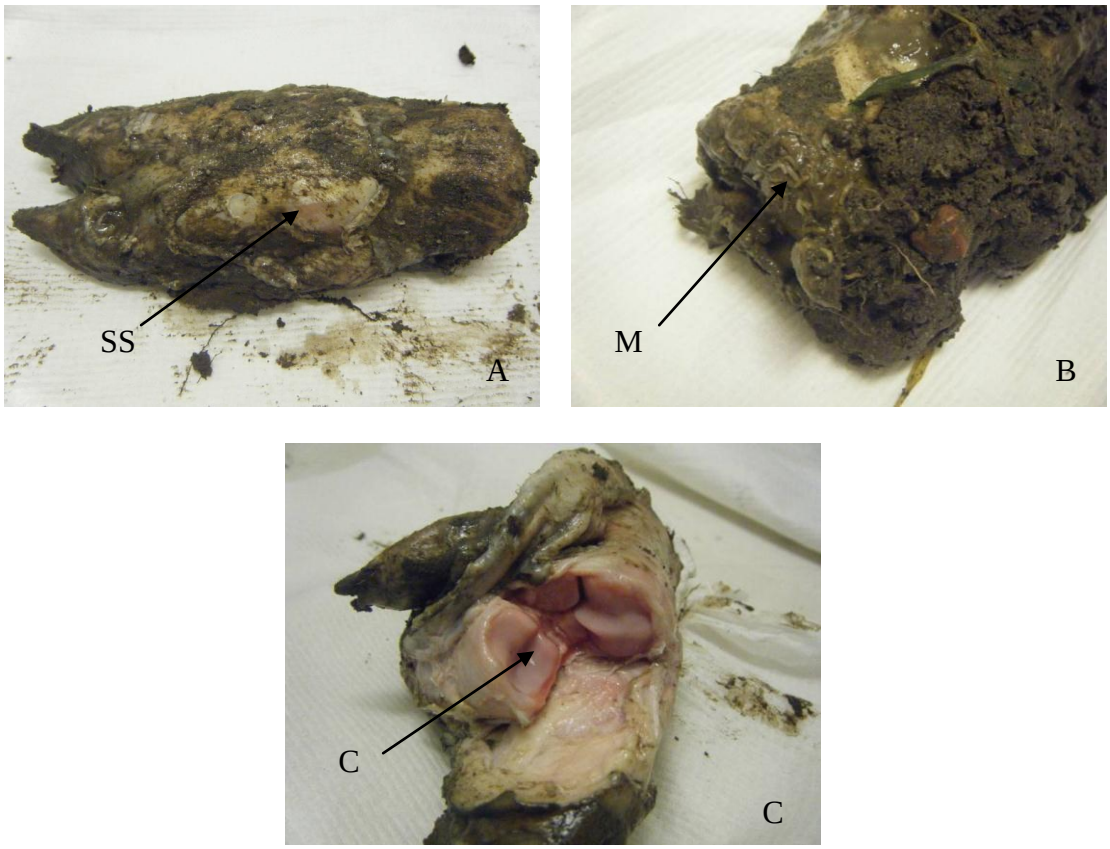


Figure 3.11: Porcine samples buried for 25 days. C: cartilage, SS: skin slippage, M: maggot activity.

28 days post burial

Externally, at 28 days post burial, the trotter exhibited a darkened appearance (Figure 3.12A). Internally the soft tissues had begun to liquefy (Figure 3.12B). Figure 3.12C shows that the joint capsule had begun to break down. The cartilage was pink in colour but was beginning to exhibit signs of loss from the articular facet (Figure 3.12D).

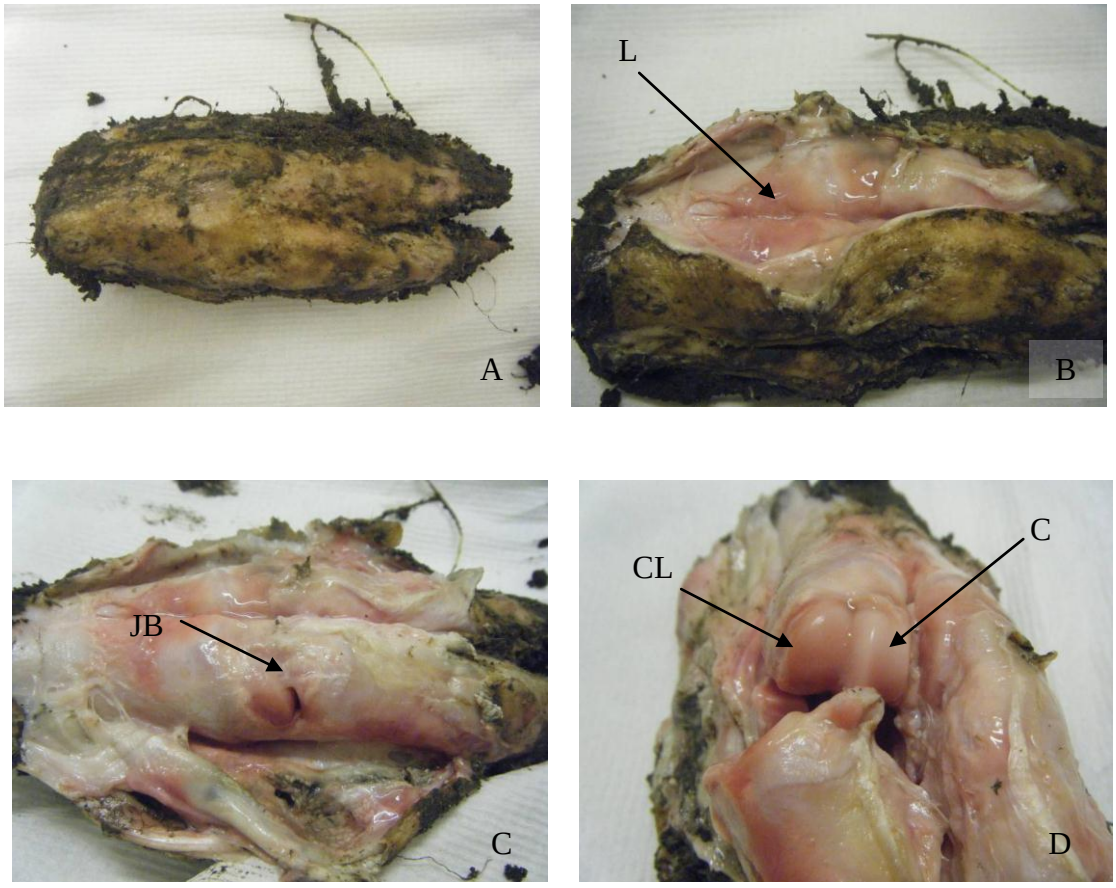


Figure 3.12: Porcine sample interred for 28 days. C: cartilage, CL: cartilage thinning, L: liquefaction, and, JB: joint capsule break down.

32 days post burial

The samples buried for 32 days could not be cleaned due to the fragility of the soft tissues (Figure 3.13A). Figure 3.13B exhibits a small area of cartilage loss with exposure of subchondral bone. The cartilage was pink in colour.

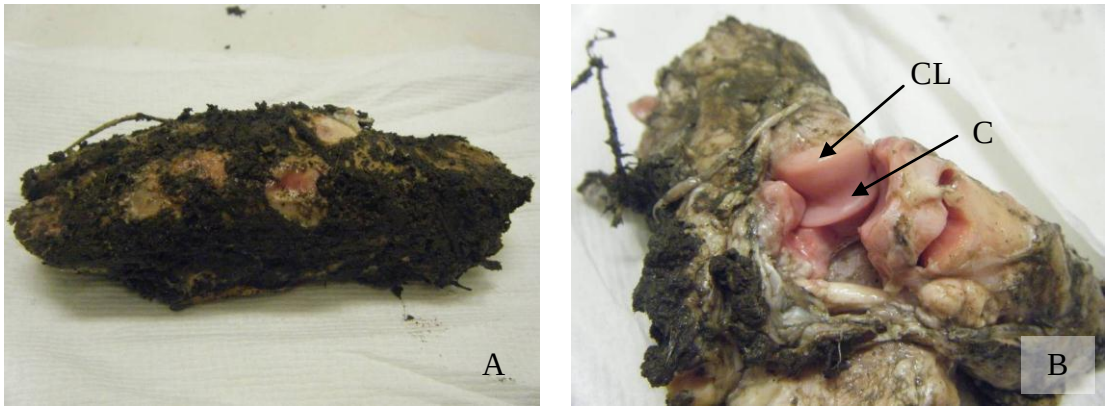


Figure 3.13: Porcine samples after 32 days burial. C: cartilage, CL: cartilage loss.

Macroscopic results 0 – 13 weeks

Figure 3.14 details macroscopic changes to cartilage from 0-13 weeks. The porcine trotters used during this dataset were interred for four, seven, 10 and 13 weeks. The control cartilage was glossy white in appearance (figure 3.14A). At four weeks postmortem the cartilage had changed from a white to a dark pink colouration (figure 3.14B). The pink colouration was evident at seven weeks, but there was a loss of cartilage from the articular facet (figure 3.14C). At 10 weeks there was extensive loss of cartilage from the articular facet, with any remaining cartilage being dull cream in appearance (figure 3.14D). Finally, at 13 weeks postmortem there were only small areas of dull cream coloured cartilage in association (figure 3.14E). Table 3.2 summarises the major macroscopic (coupled with SEM data) findings for the 0-13 week samples.

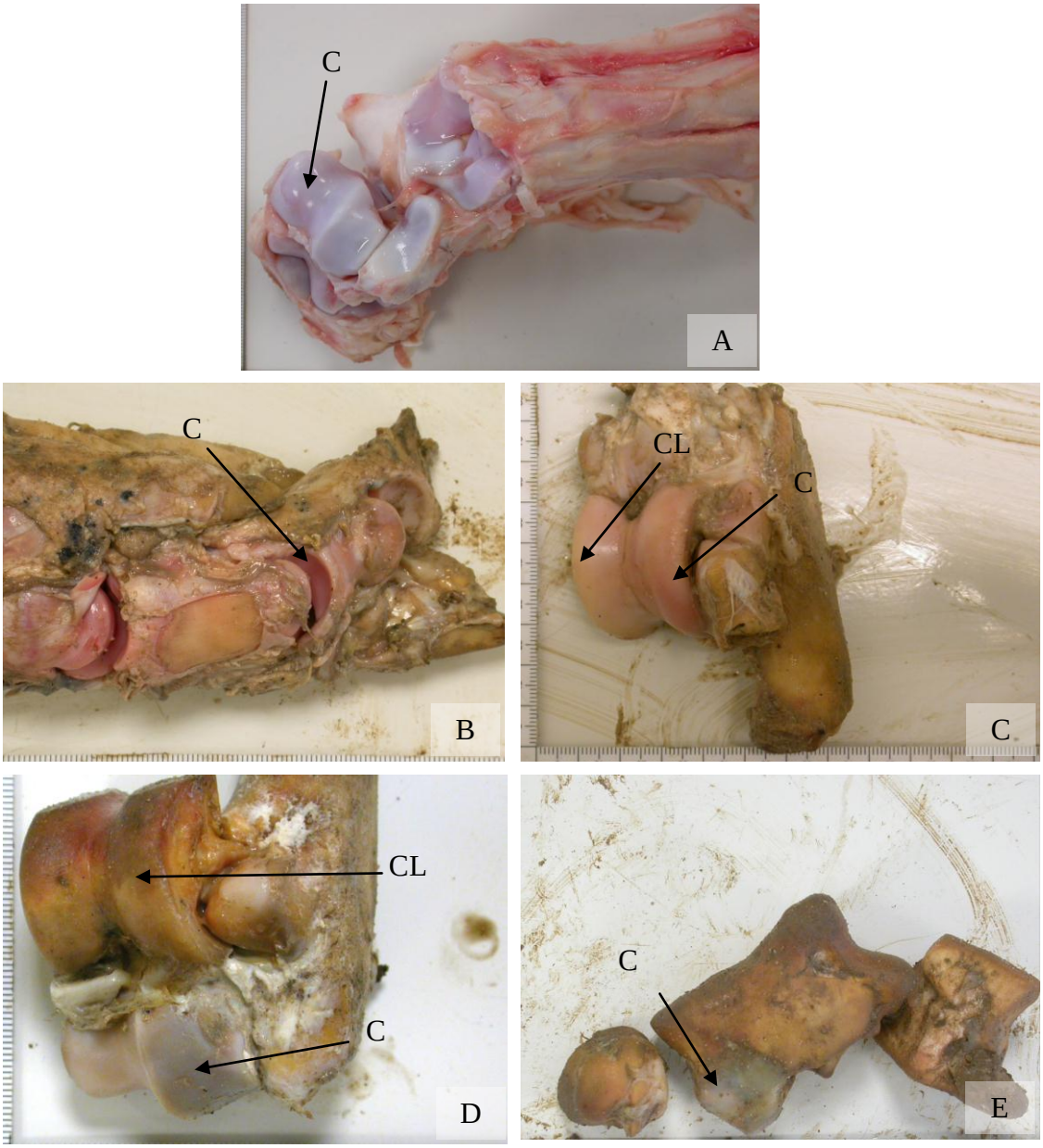


Figure 3.14: The macroscopic changes to cartilage 0 – 13 weeks postmortem. A, B, C, D and E refers to, 0, 4, 7, 10 and 13 weeks postmortem. C: cartilage, CL: cartilage loss.

There was little variation in the decomposition levels between the samples buried in the winter and summer. This indicates that the burial environment, although shallow, offered a certain degree of protection to the samples.

3.3.2 Microscopic analysis and Haematoxylin and Eosin Staining

A progressive loss of nuclei was represented in all data analysed. The following data is from replicate six. The three week sample (Figure 3.15B) was similar in appearance to the control (figure 3.15A). The dye intensity was strong and the sample was architecturally sound, and, nuclear material was in abundance (dispersed throughout the sample). The six week sample (Figure 3.15C) was, morphologically similar to the three week samples. The nine week sample (Figure 3.15D) began to exhibit signs of degradation, the dye intensity was reduced as was the level of nuclear material. The overall structure was beginning to break down with large open spaces forming. The 12 week sample (Figure 3.15E) exhibited extensive degradation with no recognisable cartilage lacunae or cells.

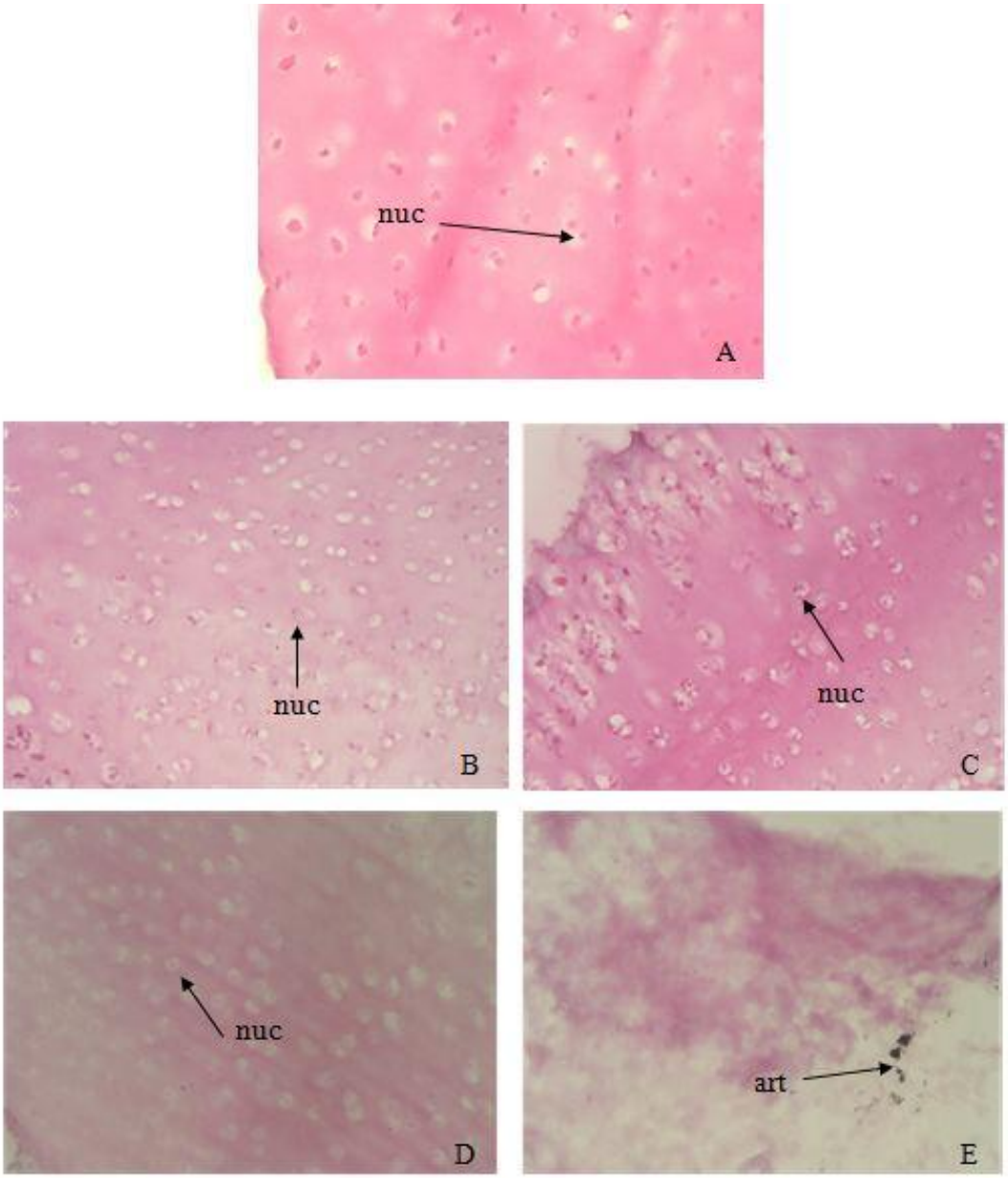


Figure 3.15: Microscopic analysis of articular cartilage. Histological sections stained with Haematoxylin and Eosin 400 x magnification. A, B, C, D and E refers to 0, 3, 6, 9 and 12 weeks. nuc = nuclear material and art = non relevant artefacts.

Additional pieces of cartilage collected from the same trotters used for the Haematoxylin and Eosin analysis were analysed with SEM which detailed changes on the cartilage surface (Figure 3.16). Similar to the macroscopic data, there was little variation in the decomposition between the samples buried in the winter and summer.

3.3.3 Scanning Electron Microscopy

The following data is from replicate six. The control cartilage (Figure 3.16A) has a smooth and intact surface with no scoring, erosions, irregularities or crystal formations. At three weeks postmortem (Figure 3.16B) orthorhombic crystals were observed. There were larger crystals (8-10 µm) present, but also smaller crystals (< 5 µm) of the same morphology in association. The overall cartilage structure was smooth, with scoring apparent. Artefacts were differentiated from crystals by SEM and SEM-EDX based on morphology and chemical composition. At six weeks (Figure 3.16C) the surface exhibited undulations with possible exposure of subsurface lacunae. Furthermore, crystals were still observable. At nine weeks (Figure 3.16D) the crystals were usually absent and the surface of the tissue was pitted and eroded. The 12 week sample (Figure 3.16E) never exhibited crystals and the tissue was extensively degraded, exposing a fibrous network. Table 3.2 summarises the SEM analysis of the cartilage surface.

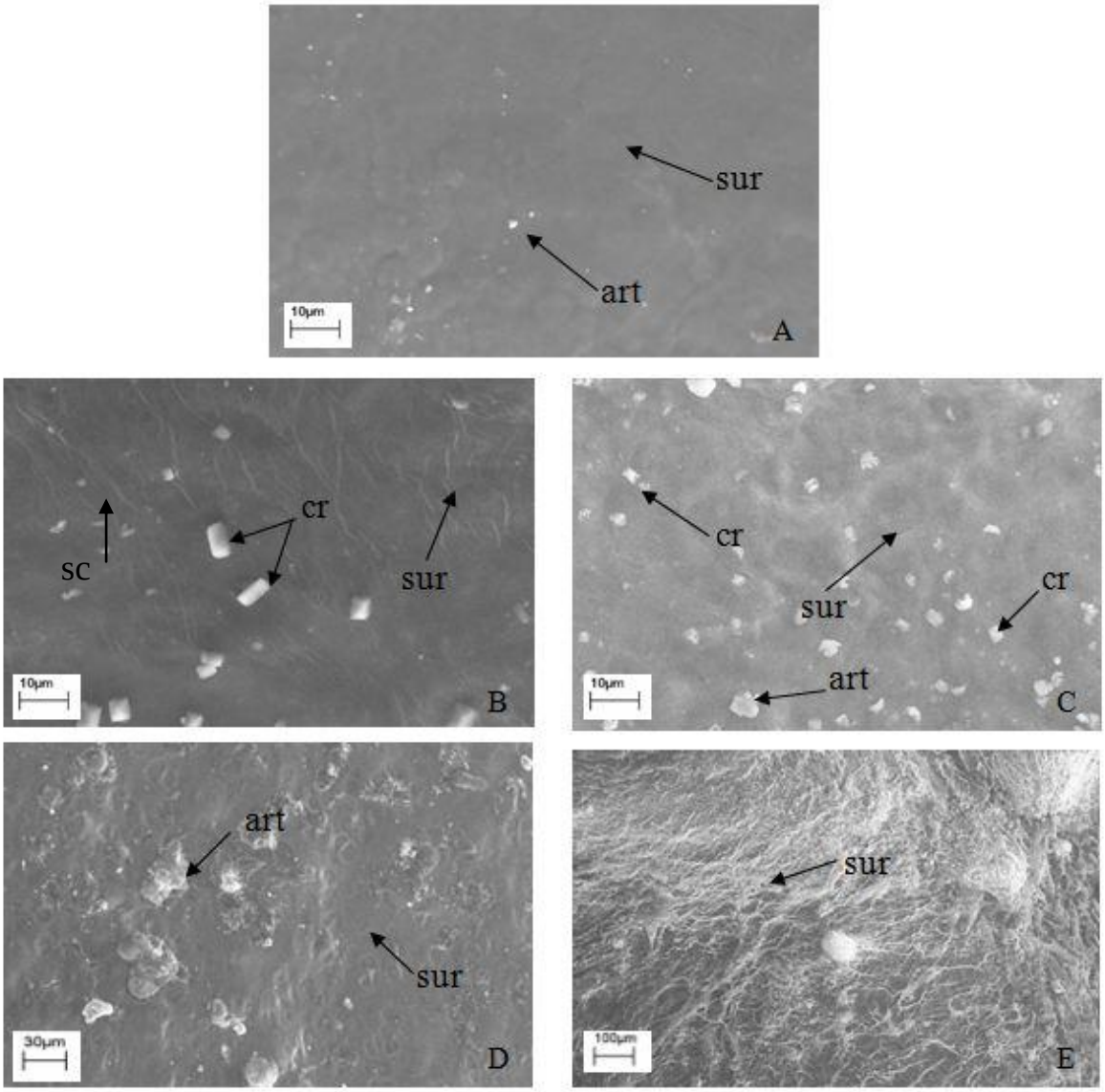


Figure 3.16: Scanning electron micrographs of articular cartilage from buried samples. A, B, C, D and E refers to 0, 3, 6, 9 and 12 weeks. Art refers to an artefact, sur to the cartilage surface, sc to scoring and and cr to a crystal.

Table 3.2: details the results obtained from macroscopic and SEM analysis of articular cartilage.

Burial Time (Weeks)	**Colour**	**Coverage of joint area**	**Texture**	**Score ( ) / Degree of articular damage. After Fuller *et al.*, (2001)**
0 (control)	Intense white and purple	Complete	Very smooth & moist	(0) no damage
4	Deep pink	Complete	Smooth	(1) minimal damage
7	Pale pink	Partial	Smooth	(2) damage to ~ 30% of cartilage
10	Dull cream	Partial	Smooth	(3) loss of 50 % of cartilage
13	Dull cream	Little	Soft	(4) severe loss of cartilage (>50%)

Table 3.2 summarises the findings of the macroscopic and SEM analysis of the 0-13 week samples. It can be seen with the control sample the cartilage has a white colouration and completely covers the joint surface. Additionally, the texture of the cartilage is smooth and retains a moist appearance due to the presence of synovial fluid. Consequently, due to the lack of observable damage to the cartilage surface it is assigned score 0 as described by Fuller *et al.*, (2001). At four weeks, the cartilage changed to a pink colour but still covered most of the joint area. The texture was still smooth but signs of damage to the surface were noted and it was assigned stage 1 (minimal damage). At seven weeks the cartilage colour changed from deep pink to light pink. The area of cartilage covering the joint decreased and there was notable damage to 30% of the cartilage surface (score 2). At 10 weeks the cartilage was cream

in colour and the joint surface was only partly covered with a loss of 50% (score 3). Finally, the 13 week sample had more than 50% cartilage loss from the joint surface. What remained was cream in colour and was assigned a score of 4.

3.3.3.1 Bacteria on the cartilage surface

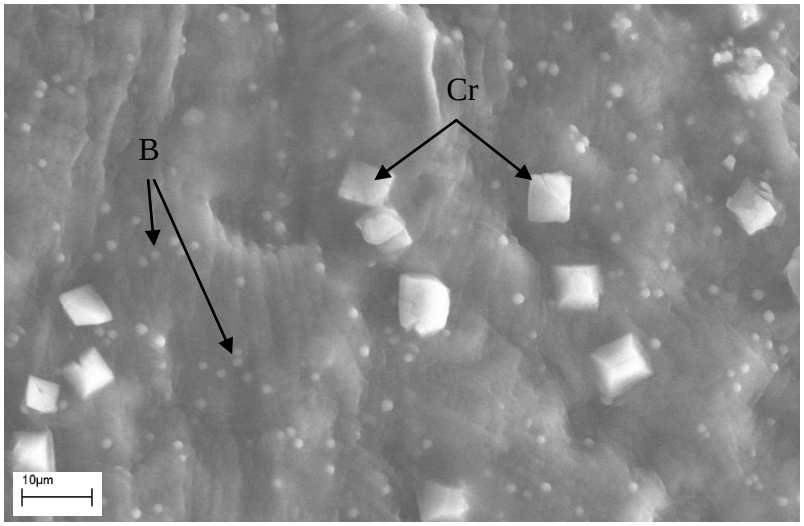


Figure 3.17: The presence of possible cocci bacteria on the cartilage surface is close proximity to the crystals. B: bacteria, Cr: crystals.

While conducting SEM analysis of the cartilage surface small round structures of approximately 1 µm in diameter were observed in close proximity to the crystal formations (figure 3.17). These circular structures had the morphological appearance of cocci shaped bacteria. The abundance of the possible bacteria had no observable correlation with the quantity of the crystals.

3.3.4 Bacterial culture on B-41 media

Control cartilage was inoculated on B-41 medium but yielded no bacterial growth after three days incubation at 30ºC (figure 3.18A). This demonstrated that the control cartilage was a sterile tissue. However, cartilage from the previously interred 28 days sample (also inoculated on B-41 medium) exhibited bacterial growth after three days of incubation at 30ºC. The bacterial colonies grow in accordance with the streak pattern adopted to inoculate the plate (figure 3.18.B). A section of cartilage is located in the centre of each of the plate.

Although bacterial colonies were observed after three days of incubation, crystal structures were not seen until the fourth day as shown in figure 3.19.

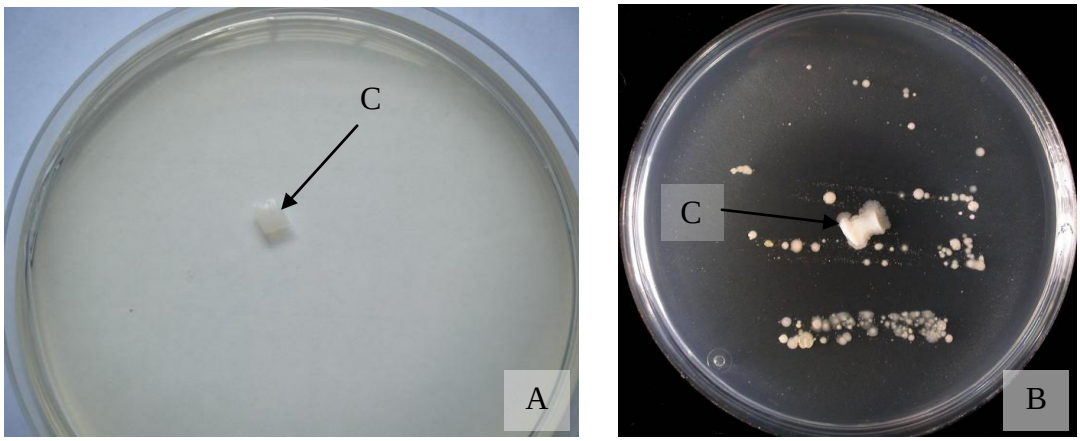


Figure 3.18: Bacteria from articular cartilage inoculated on B-41 agar. 3.18A shows the control cartilage with no bacterial growth. 3.18B shows bacterial growth after three days of incubation with the bacterial colonies following the streak pattern adopted to inoculate the plate. C: cartilage.

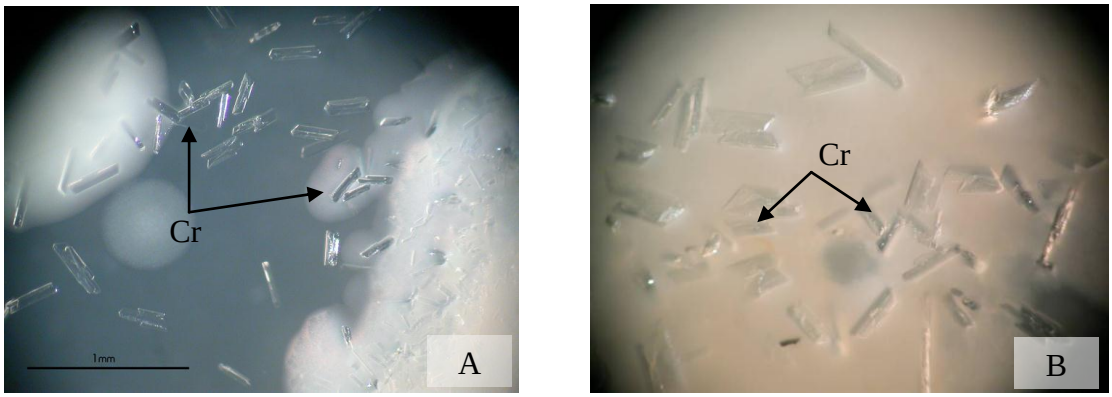


Figure 3.19: Crystal formations. The cream coloured structures are the bacterial colonies. Figure 3.19A shows sample four days after plating, figure 3.19B shows growth after six days. Cr: crystals.

Figure 3.19 shows the typical crystal formation from the cultured bacteria. The crystals are approximately 0.5 mm in length with a rod shaped appearance. The terminus of each crystal exhibits a tapered morphology (orthorhombic). The crystals were visible after four days of incubation and their morphology strongly correlated with the crystals documented on the cartilage surface. However, the crystals produced on the B-41 media are significantly larger than those on the cartilage surface (0.5 mm and 5-10 µm respectively). There was a correlation between time and amount of crystals present.

However, in addition to the orthorhombic crystals, further structures were documented. These were needle like in shape in appearance and travelled inferiorly into the agar (figure 3.20).

This made them fragile and difficult to extract from the agar matrix and consequently analysis of this particular crystal precipitation ceased at this point.

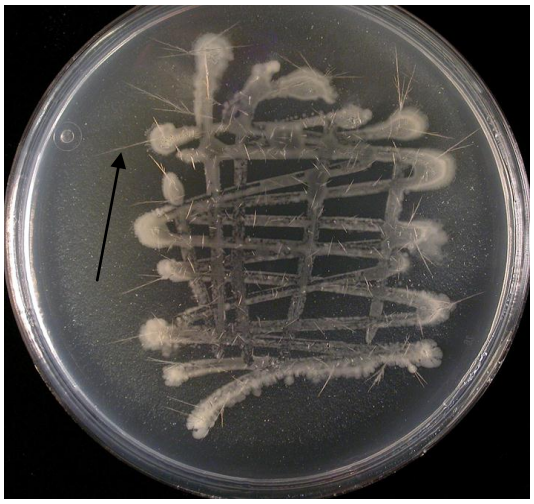


Figure 3.20: Bacterial growth resulting in needle shaped crystal precipitation – highlighted by arrow.

In addition to the bacteria shown above, a further species was cultured. This bacterial species grew both on and within the agar. It had a fluffy morphology different to the species shown in figures 3.18 and 3.19, and seemingly formed small circles as it developed (figure 3.21). This species of bacteria did not produce any crystals.

Figure 3.21: A further species of bacteria cultured from cartilage postmortem, it has a fluffy morphology. Note: the small circular clusters of bacteria are contamination.

The crystals produced by bacteria appeared to form within the colonies as shown in figure 3.22. However, they appear to be expelled from the colonies onto the agar matrix (figure

3.22A, B and C). It was also apparent the crystals could form as single structures but also as clusters as seen in figure 3.22C.

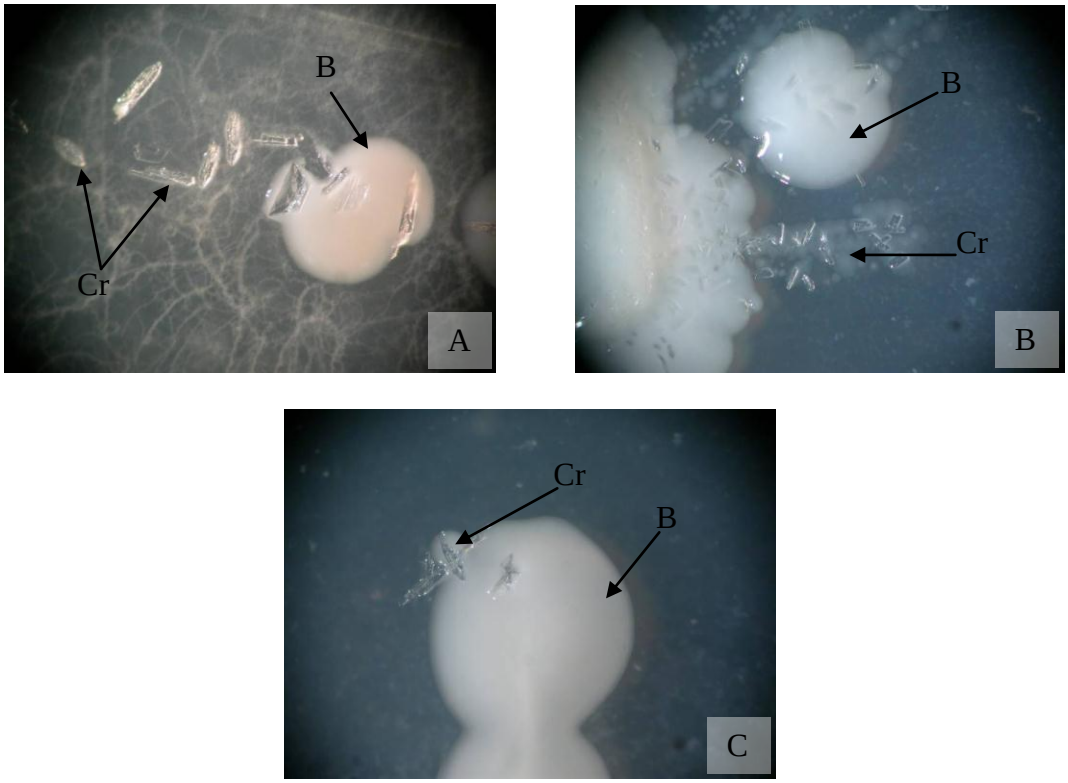


Figure 3.22: Precipitation of crystals within bacterial colonies and their possible expulsion onto the agar matrix. 3.22 A and B show individual crystal formation, whereas 3.22C show crystal clusters. B: bacteria, Cr: crystals.

SEM analysis was conducted to determine if the crystals precipitated from the bacteria were morphologically similar to those seen on the porcine cartilage postmortem (figure 3.23). The crystals were morphologically similar (figure 3.23C).

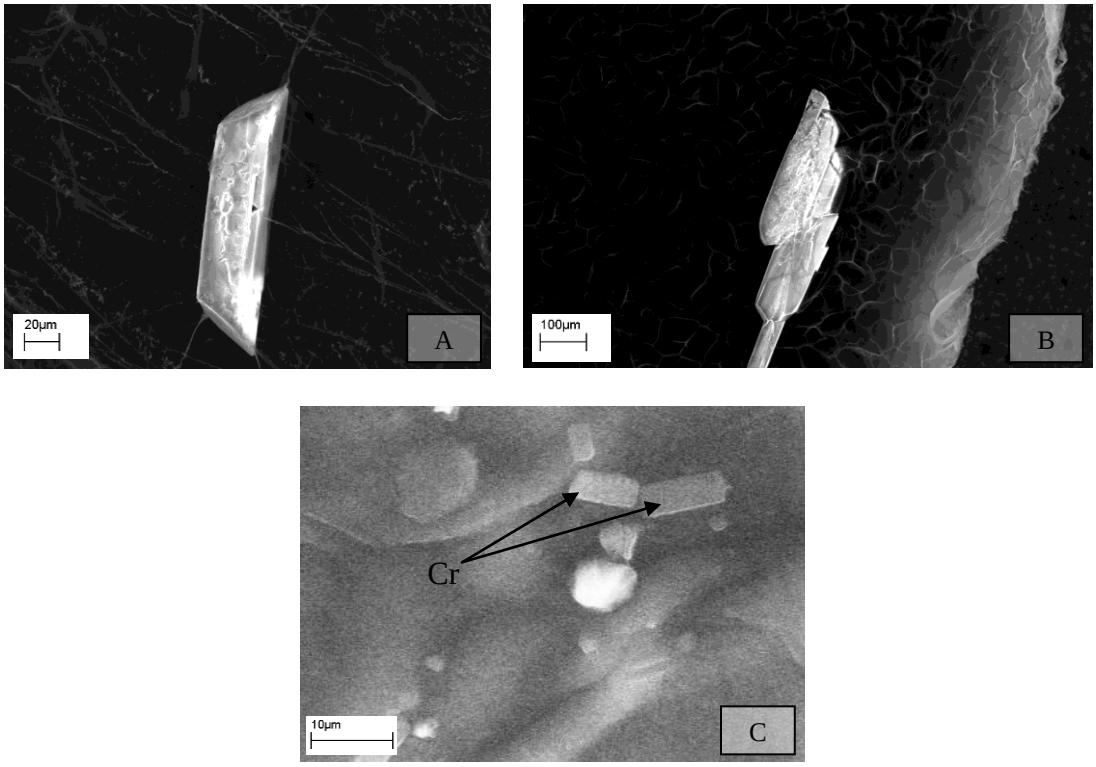


Figure 3.23: SEM analysis of the crystals present on the B-41 agar compared with crystals located on porcine cartilage surface. Image 3.23A shows a large single crystal whilst image 3.23B shows a small cluster. Figure 3.23C shows crystals from the porcine cartilage surface. Cr: crystals.

3.3.5 Scanning Electron Microscopy and Energy Dispersive X-ray Analysis (SEM-EDX) of crystal

SEM-EDX analysis was conducted on the crystals from the cartilage surface and those precipitated from the bacterial colonies to determine their elemental composition (tables 3.3 and 3.4). The crystal composition indicated that they are made of numerous elements with carbon, nitrogen, oxygen, magnesium and phosphorous being the five dominant constituents.

Table 3.3: Results for the Scanning electron microscopy coupled with energy dispersive x-ray (SEM-EDX) analysis of the rectangular crystals observable on the surface of articular cartilage postmortem. Dominant elements are highlighted in bold.

Element	Weight %
C K	**29.05**
N K	**14.57**
O K	**46.48**
Na K	1.66
Mg K	**2.86**
P K	**3.01**
S K	0.24
Cl K	2.01
Ca K	0.12

Table 3.3 details the elemental composition of the postmortem crystals located on the cartilage surface. The five dominant elements include carbon, nitrogen, oxygen, magnesium and phosphorus. Additional elements found within the crystals were sodium, sulphur, chlorine and calcium.

Table 3.4: SEM-EDX analysis for the crystals harvested from the bacterial colonies. Dominant elements are highlighted in bold.

Element	Weight %
C K	**14.35**
N K	**7.71**
O K	**58.70**
Na K	0.18
Mg K	**6.89**
P K	**9.27**
S K	0.20
K K	2.89

Table 3.4 shows the elemental composition of the crystals precipitated from bacterial colonies. The dominant elements found within these crystals included carbon, nitrogen, oxygen, magnesium and phosphorus. Additional elements included sodium, sulphur and potassium.

The two presented tables show that both the crystals from the cartilage surface and the bacterial colonies share the same five dominant elements (carbon, nitrogen, oxygen,

magnesium and phosphorus). The levels of magnesium and phosphorus are higher in the crystals produced on the B-41 media compared to the crystals on the cartilage, this could be due to higher concentrations of magnesium and phosphorus within the media, or, a larger crystal size with the crystals produced by the bacteria. The crystals precipitated by the bacteria contained potassium probably as a result of bacterial metabolism on a medium containing dipotassium hydrogen phosphate. The crystals from the cartilage surface contained chlorine and calcium which were absent in the crystals produced by bacteria.

The SEM-EDX data collected was compared to SEM-EDX analysis conducted on a sample of manmade (synthetic) struvite (table 3.5) synthesised and obtained from chemistry lab technicians.

Table 3.5: SEM -EDX data for synthetic struvite. The dominant elements are highlighted in bold.

Element	Weight %
C K	**28. 12**
N K	**5.62**
O K	**42.28**
Na K	0.52
Mg K	**9.73**
P K	**13.73**

The manmade struvite contained the same five dominant elements (table 3.5) as seen with the crystals from the cartilage surface and bacterial colonies (tables 3.3 and 3.4). However, fewer additional elements (only sodium) were identified in the manmade struvite compared with the crystals found on the cartilage and precipitated by the bacteria (sulphur, chlorine and calcium, and, sulphur and potassium respectively).

To determine how the elements were positioned within the crystals on the cartilage surface a mapping application of the SEM-EDX was implemented as shown in figures 3.24 – 3.27. This process allows for element distribution to be determined within a sample. Oxygen, magnesium and phosphorus are highlighted as red, blue and purple colours respectively within the sample.

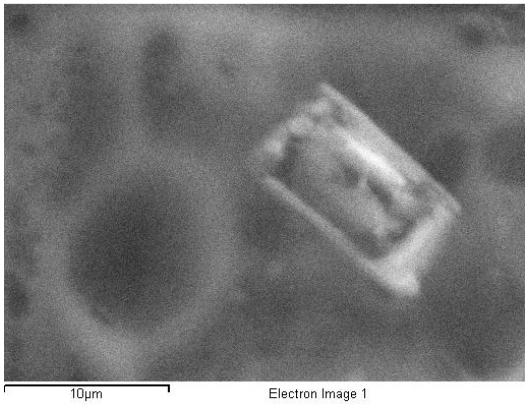


Figure 3.24: An individual crystal located on cartilage surface before EDX mapping.

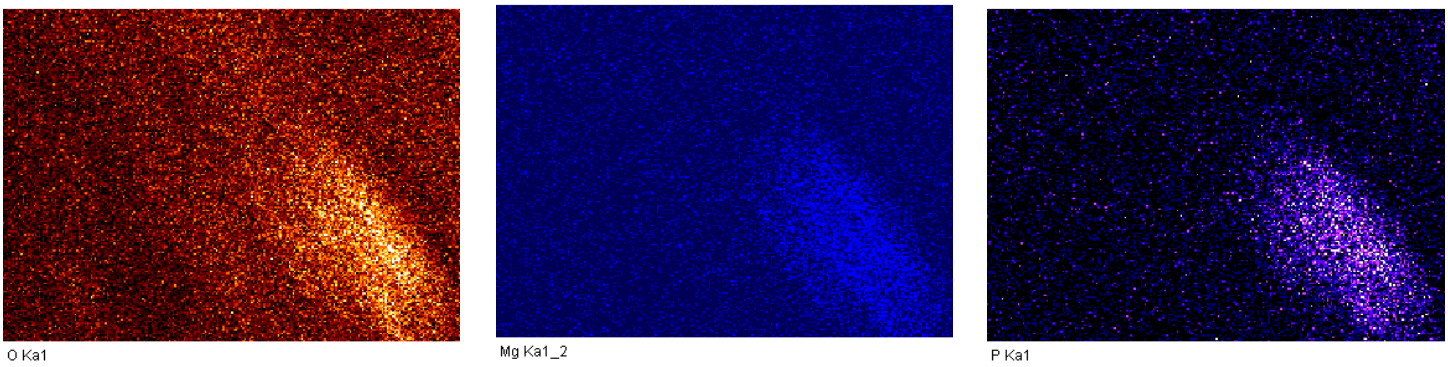


Figure 3.25: Results of EDX mapping, the left (red), middle (blue) and right images (purple) show the positioning of oxygen, magnesium and phosphorus elements within the sample. There is slight blurring to the sample moving during analysis.

Figure 3.23 demonstrates that oxygen, magnesium and phosphorus were widely distributed within the crystal structure. There was also a wide distribution of the aforementioned elements within the cartilage surface suggesting that the background matrix also contained a high abundance of these elements.

The same analysis was conducted on small groups of crystals as seen in figure 3.26 and 3.27.

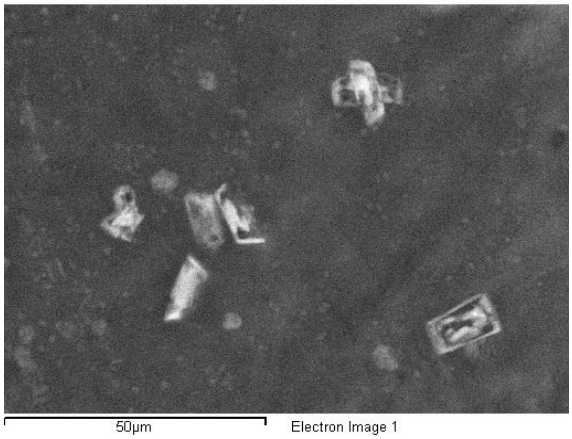


Figure 3.26: A collection of crystals on the cartilage surface before EDX mapping.

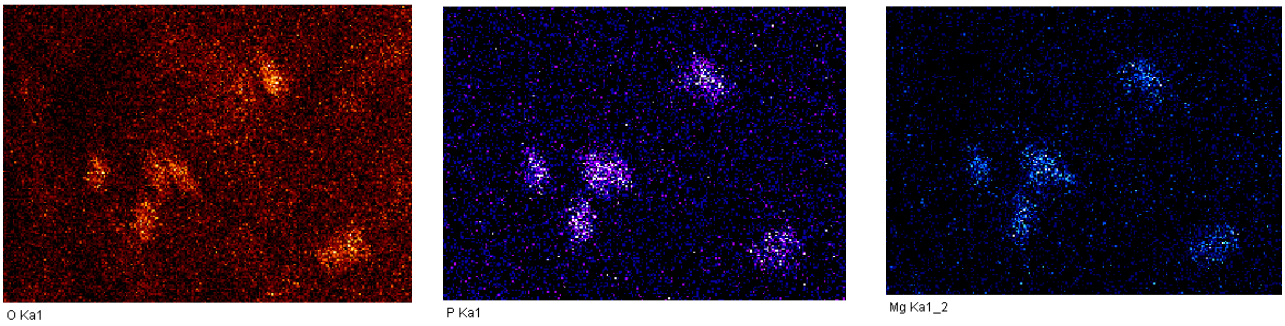


Figure 3.27: EDX mapping analysis of a small group of crystals. The left (red), middle (blue) and right images (purple) show the positioning of oxygen, magnesium and phosphorus elements within the sample.

Figure 2.27 shows a small group of crystals on the articular surface. It can be seen that all crystals contain oxygen, magnesium and phosphorus throughout their individual structures. This data also highlighted the abundance of these elements within the cartilage surface.

3.3.6 Influence of soil on crystal formation?

To determine if contact with soil was responsible for crystal formation trotters were stored in sealed plastic containers placed outside during the summer. This was an additional experiment which subjected the porcine samples to conditions that differed from the burial conditions utilised for the main study. The containers were sealed to limit access for insects and scavengers and also to exclude the process of mummification which would hinder subsequent dissection.

Crystals were found to form on the cartilage surface after seven days in July (figure 3.28). This data indicates that these crystals do not form as a result of soil interaction. However, under these conditions the crystals formed within seven days compared to three weeks in a burial setting. This suggests that variables such a temperature influenced their developmental speed. Crystals of numerous sizes were noted on this sample.

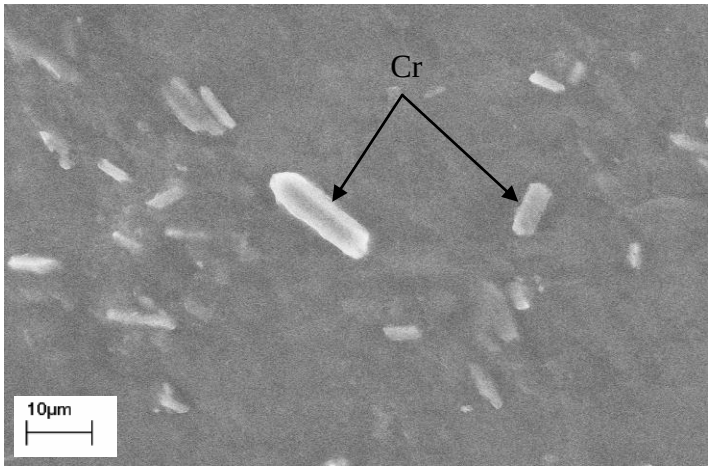


Figure 3.28: SEM image of postmortem rectangular crystals observed on cartilage in sealed plastic containers, outside of a soil environment. Note the crystals of different sizes.

3.3.7 Analysing for magnesium in cartilage sections using Titan Yellow

Since the SEM-EDX data confirmed that magnesium was a major component in the crystals structure, a histological stain specific to magnesium, Titan yellow, was applied to cartilage sections to see if crystals (of the already discussed morphology) were present within the cartilage tissue (Figure 3.29A and B). Titan yellow was yellow / orange in colour when wet (figure 3.30A).

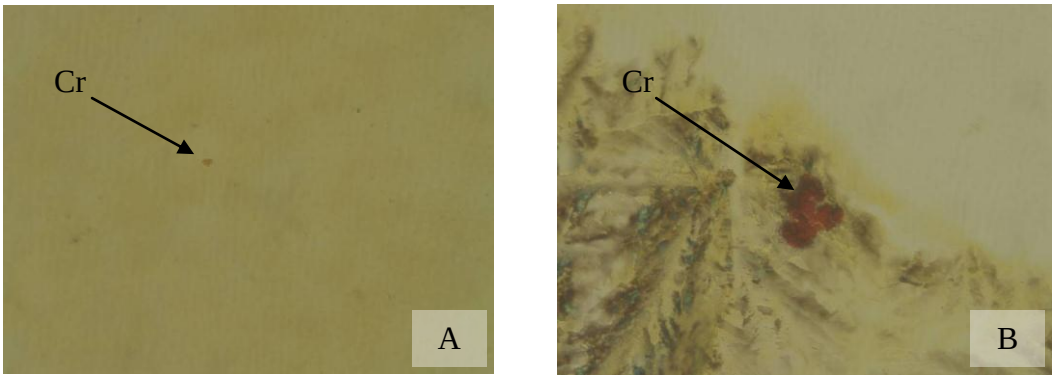


Figure 3.29: Cartilage sections at six weeks postmortem stained with Titan Yellow. Figure 3.29A shows an individual red crystal whereas Figure 3.29B shows the crystals in a cluster. 400x magnification. Cr: crystals.

Crystalline structures were noted within the cartilage sections when subjected to light microscopy. However, these did not correlate with the orthorhombic morphology as demonstrated with the crystals on the cartilage surface. It was noted that the Titan Yellow, which should turn red in the presence of Magnesium, actually turned red when dried and without contact with Magnesium (figure 3.30B). This resulted in a false positive for the presence of magnesium, consequently the application of Titan yellow proved to be inaccurate for this analysis. Thus the positive results seen in figures 3.29 A and B were rejected.

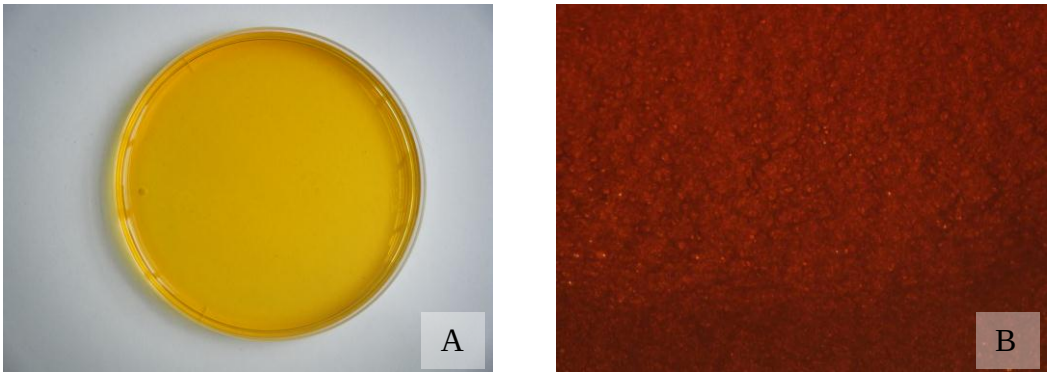


Figure 3.30: Titan yellow in a wet and dry state (figure 3.30A and B respectively).

3.3.8 X-Ray Diffraction Analysis of Postmortem Crystals

Figure 3.31 A, B and C shows the XRD spectra for cartilage that contained the crystals. The vertical axis is the intensity of reflection and the horizontal axis (2 theta), refers to the scattering angle for that reflection (Pickworth Glusker and Trueblood, 1972). Two broad peaks were noted at the far left of each spectrum at 8-10 theta and 18-22 theta (see arrows). However, each of the spectra appeared different, possibly due to the orientation of the crystals in the instrument during analysis resulting in differences in diffraction of the X-rays. Unfortunately, due to the small crystal size (approx. 10 µm) the crystals could not be reoriented by the analyst to ensure that all faces of the crystals could be analysed. Additional to the small crystal size, the quantity of crystals on the cartilage surface may have also influenced the results as the quantity would have varied between the samples.

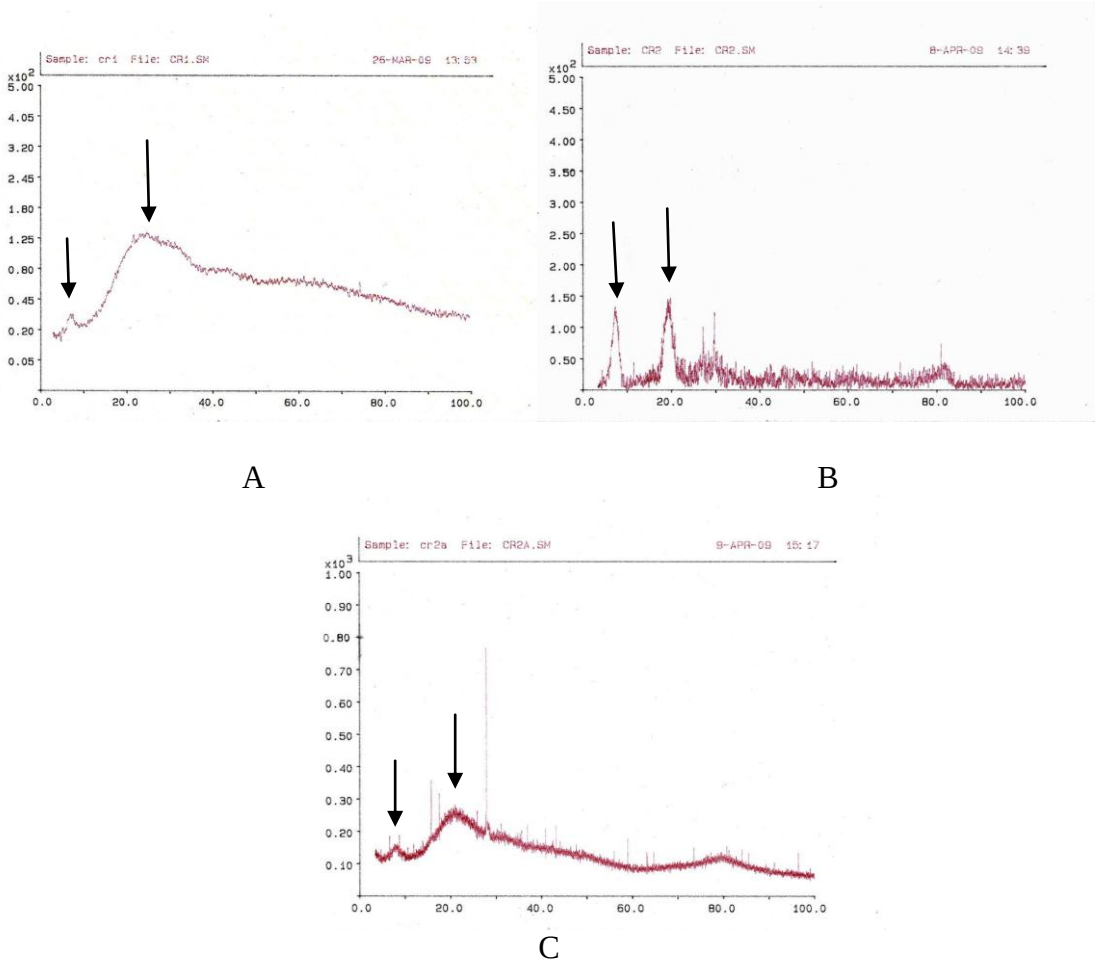


Figure 3.31: XRD analysis of the crystals growing on the cartilage surface. The arrows indicate 8-10 theta and 18-22 theta respectively.

After the successful precipitation of crystals by bacteria on the B-41 medium they were harvested and subjected to XRD analysis (figure 3.32).

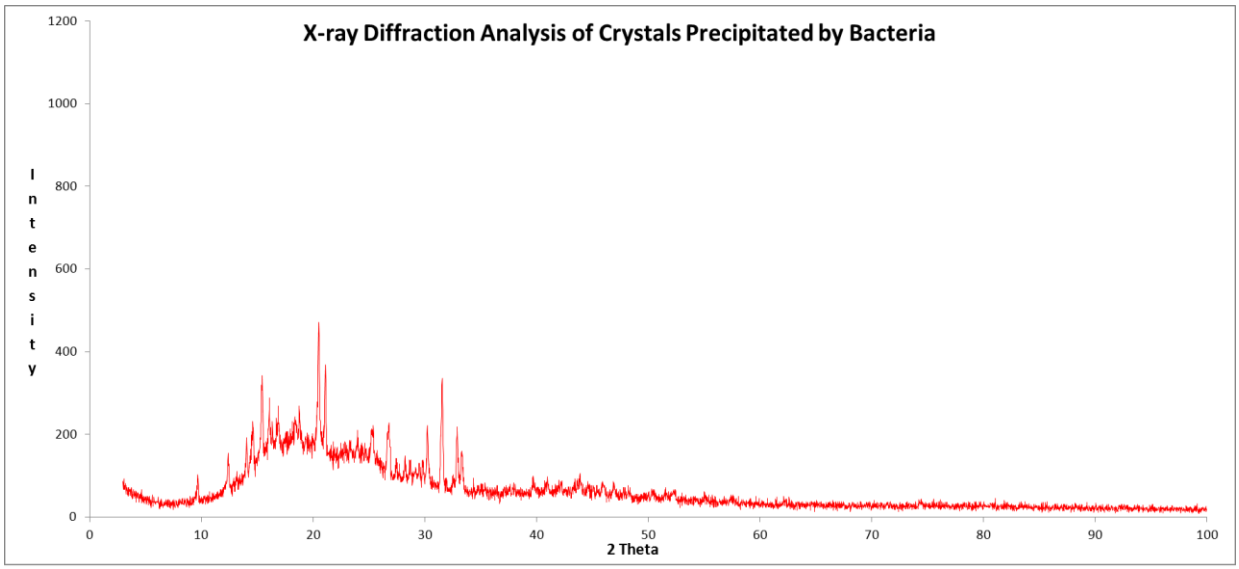


Figure 3.32: XRD analysis of crystals collected from bacterial colonies innoculated on B-41 media.

The XRD analysis of the crystals from bacterial colonies exhibited some clear, explicit peaks although the X-ray intensity (vertical axis) was low. This spectrum was compared to an XRD spectrum from the same synthetic struvite that was utilised for the SEM-EDX analysis (figure 3.33).

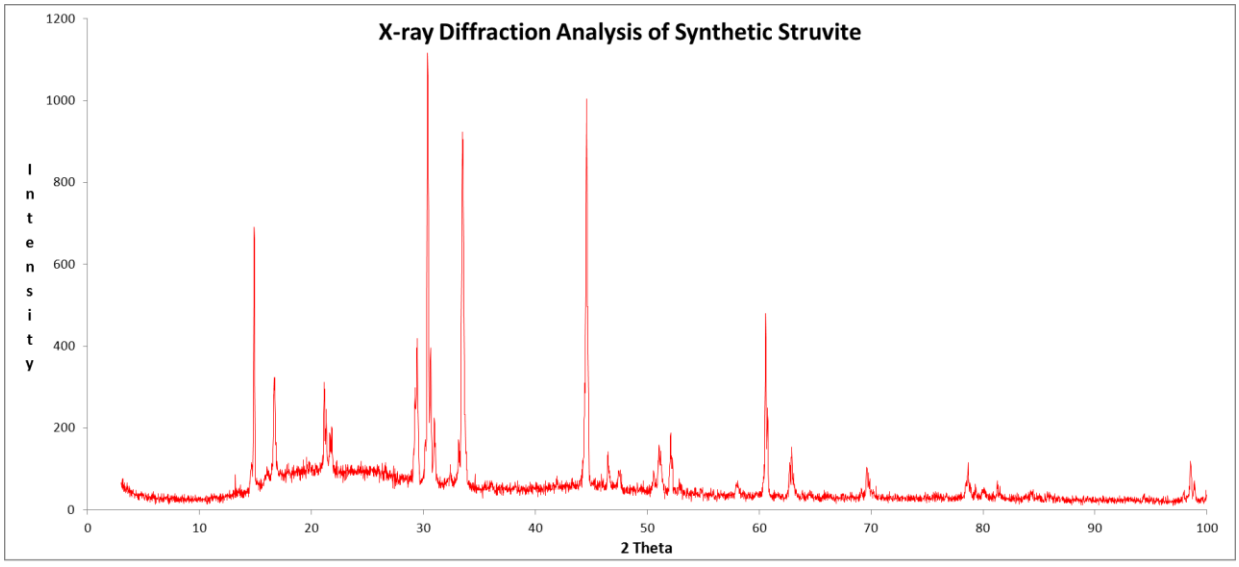


Figure 3.33: XRD analysis of synthetic struvite.

The results collected from the XRD do not obviously match with the peak intensities on the synthetic struvite higher than the spectrum from the bacterial crystals. The two obtained spectra were overlaid to look for areas of similarity (Figure 3.34).

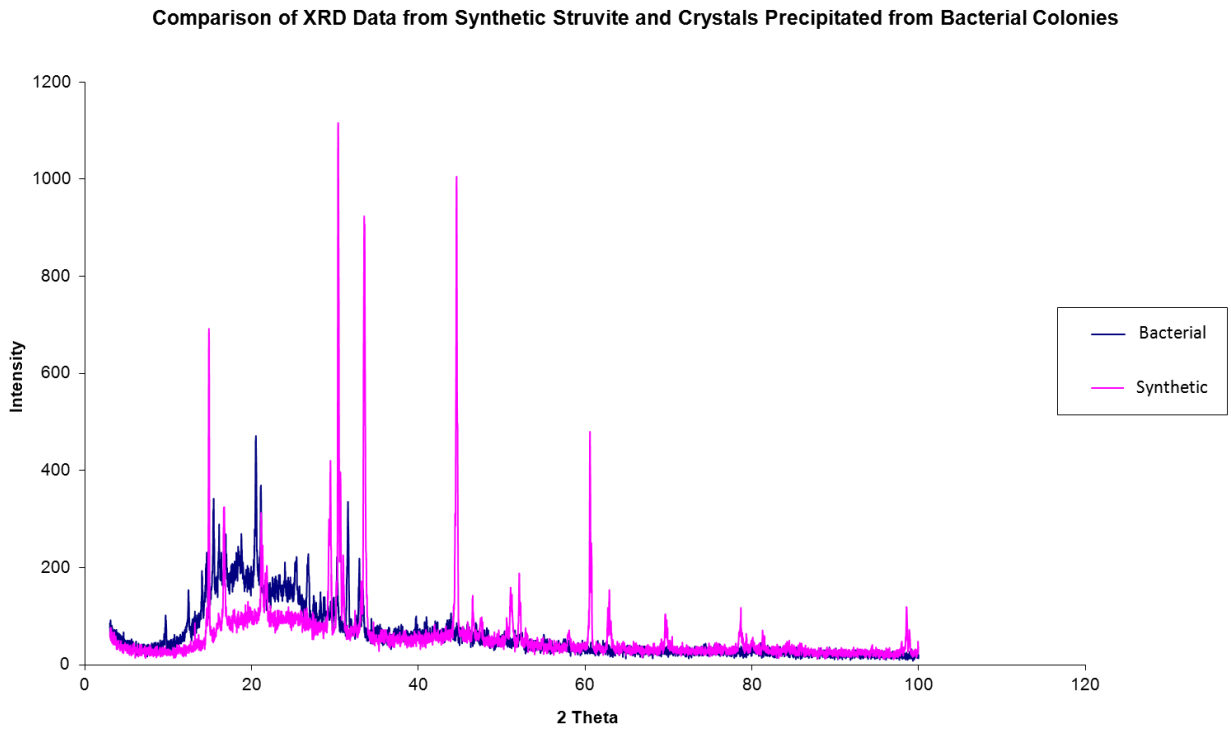


Figure 3.34: XRD comparison of synthetic struvite and crystals precipitated by bacterial colonies.

It was found that there were some similarties with the XRD data obtained from synthetic struvite and crystals precipitated by the bacterial colonies. There are matching clusters between both samples at 15-17 theta, 21 theta and 30-35 theta. However, the bacterial crystals exhibited no discernable peaks after 50 theta resulting in no further matches between the samples.

3.3.9 pH change on the articular surface of cartilage

A pH change was documented on the surface of the articular cartilage as shown in Figure 3.35. This data set included burial intervals of between four and 42 days. Two trotters were analysed per postmortem interval. The control cartilage demonstrated a pH of 7. However, at 25 days postmortem the cartilage became alkaline at pH 8. The time at which this pH change occurred differed by a few days in the five replicates conducted. However, such pH changes always occurred after 21 days postmortem (range 22-25 days). The time of the pH change to

the cartilage surface can be directly linked to the precipitation of crystals, suggesting that the precipitation of the crystals is linked to the change in pH. In this instance, it would suggest that at an alkaline pH the crystals form and are present on the cartilage surface, whereas at a neutral pH the crystals are absent. The impact of this finding is discussed in chapter 3.4. Table 3.6 shows data for the burial intervals, pH of the cartilage surface and the presence of crystals for all five replicates.

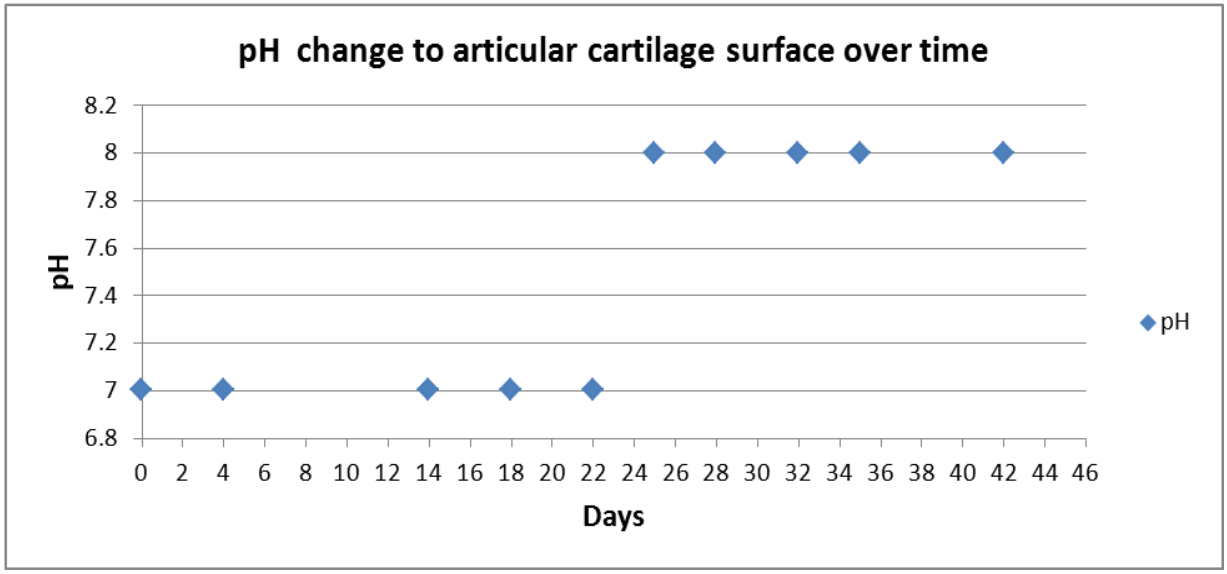


Figure 3.35: The pH change to the articular cartilage surface over time. Burial intervals ranged from four to 42 days. For this sample set, two trotters were analysed per postmortem interval.

Table 3.6: Change in pH of articular cartilage and correlation with precipitation of crystals. Rep: replicate number, N: no crystals present, Y: crystals present, NA: not applicable. For replicate dates please refer to table 3.1.

	Burial Interval (Days)															
Rep : 2	27	49	70	91												
pH	8	8	8	NA												
Crystals	Y	N	N	N												
Rep: 3	4	11	14	18	22	25	28	32	35	42						
pH	7	7	7	7	8	8	8	8	8	8						
Crystals	N	N	N	N	Y	Y	Y	Y	Y	Y						
Rep: 4	7	13	15	20	23	27	30	33	37	45	48					
pH	7	7	7	7	8	8	8	8	8	8	N/A					
Crystals	N	N	N	N	Y	Y	Y	Y	Y	Y	N					
Rep: 7	3	6	9	12	15	17	20	23	26	29	32	35	39	42		
pH	7	7	7	7	7	7	7	8	8	8	8	8	8	8		
Crystals	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y		
Rep: 8	2	5	8	10	13	16	19	22	24	26	30	33	36	40	44	46
pH	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8
Crystals	N	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	N

Table 3.6 details the pH changes to the cartilage surface and the probable correlation with the precipitation of crystals. When at a neutral pH (pH 7) crystals were absent from the cartilage surface. However, when the pH elevelated to 8, crystal precipitation was noted. In replicate two, at 13 weeks, little cartilage was available for analysis and pH data was not collected. In certain instances, replicate four at 48 days, and replicate eight at 46 days, crystals were absent from the cartilage surface. Statistical assessment of this data using Chi^2 analysis highlighted that the relationship between pH and presence of crystals on the cartilage surface was highly significant ($P<0.0001$).

3.3.10 Disappearance of crystals from the cartilage surface

By comparing the micrographs with the macroscopic images it was noted that crystals were present on cartilage from joints which were fully articulated and where the cartilage was protected by soft tissue (figure 3.35A and B). Conversely, crystals were absent from open

joints, where the cartilage was exposed due to being compromised by the decomposition process (figures 3.35C and D)

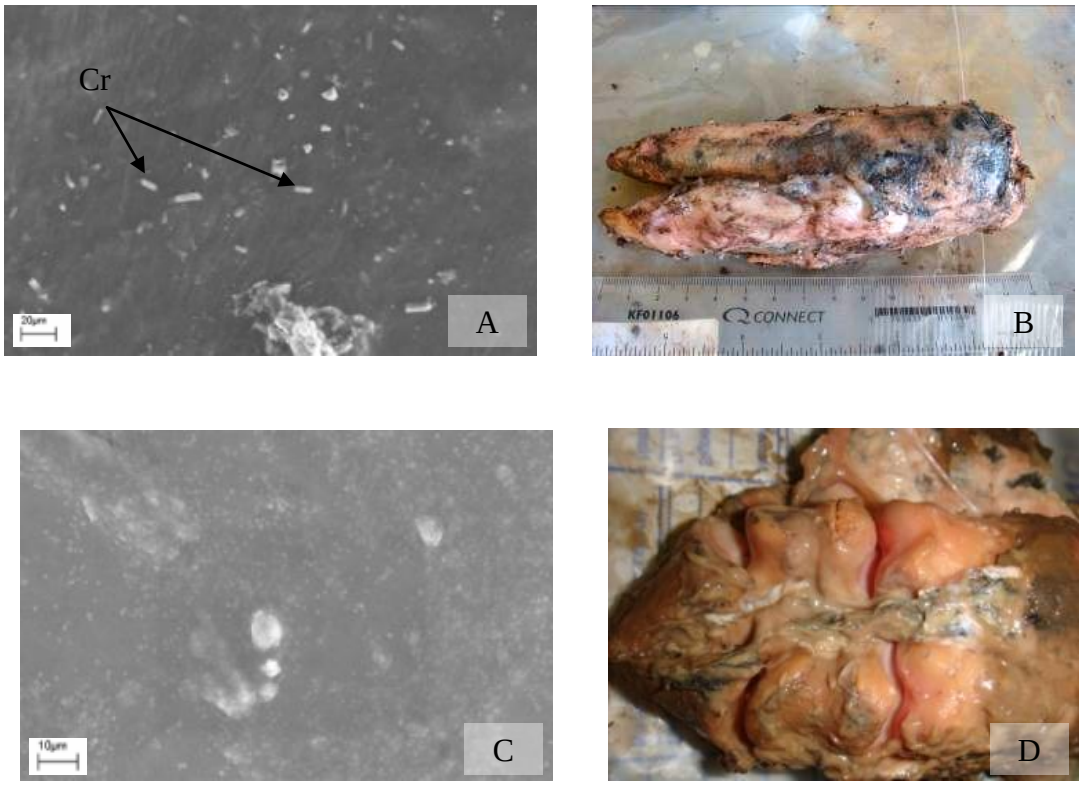


Figure 3.35: The possible correlation of the level of decomposition with the dissapearance of the crystals. Figure 3.35A shows the crystals present on an intact and articulated trotter (figure 3.35B), while figure 3.35C illustrates no crystals on the disarticulated trotter (Figure 3.35D). Cr: crystals.

Additionally, when crystals were treated with rainwater percolated through Compton soil the crystals dissolved (figure 3.36A). However it was found that when treated with neutral distilled water the crystals persisted (figure 3.36B).

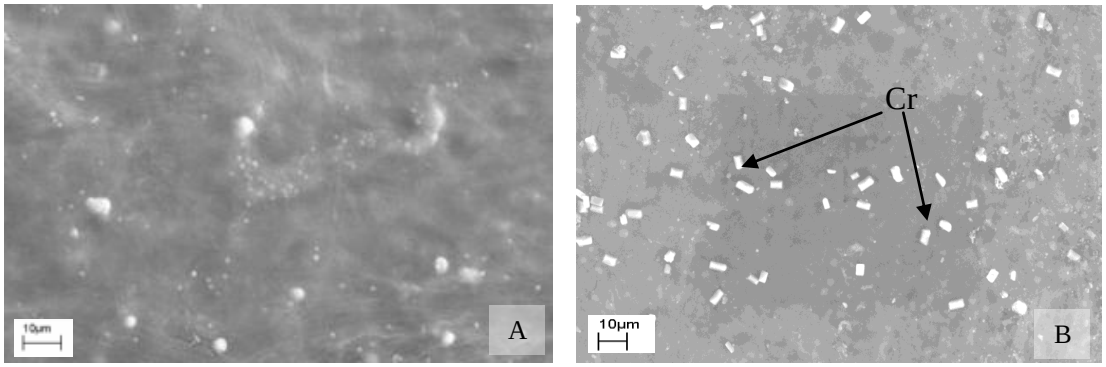


Figure 3.36A: Cartilage after application of acidic rain water compared to cartilage treated with neutral distilled water (figure 3.36B). It can been seen that the crystals are absent. Cr: crystals.

These findings suggest that acidic rain water (average 5.6) when percolated through acidic Compton soil has a detrimental effect on the crystals. These crystals were not displaced by the water, but were seen to actually dissolve in the rain water solution.

3.3.11 Identification of bacteria species from cartilage

Three species of bacteria were presumptively identified from GC-MS analysis of their fatty acids. This analysis was kindly performed by Zofia Piotrowska-Seget at the Uniwersytet Slaski, Poland. The suggested identifications were: *Acinetobacter calcoaceticus, Acinetobacter iwoffii* and *Grimontia hollisae*.

DNA analysis conducted by NCIMB Ltd successfully identified a crystal producing bacteria as a *Comamonas* species across 100% of the entire DNA sequence.

The DNA sequence generated was:

GATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGTAACAGGTCTTC
GGATGCTGACGAGTGGCGAACGGGTGAGTAATACATCGGAACGTGCCTAGTAGT
GGGGGATAACTACTCGAAAGAGTAGCTAATACCGCATGAGATCTACGGATGAAA
GCAGGGGATCGCAAGACCTTGTGCTACTAGAGCGGCTGATGGCAGATTAGGTAG
TTGGTGGGATAAAAGCTTACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGACG
ATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGCAGGATG
AAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAGCTTTGGGTTAATA
CCCTGGAGTCATGACGGTACCGTAAGAATAAGCACCGGCTAACTAC

Input of this sequence into BLAST allowed for a phylogram to be generated (figure 3.37).

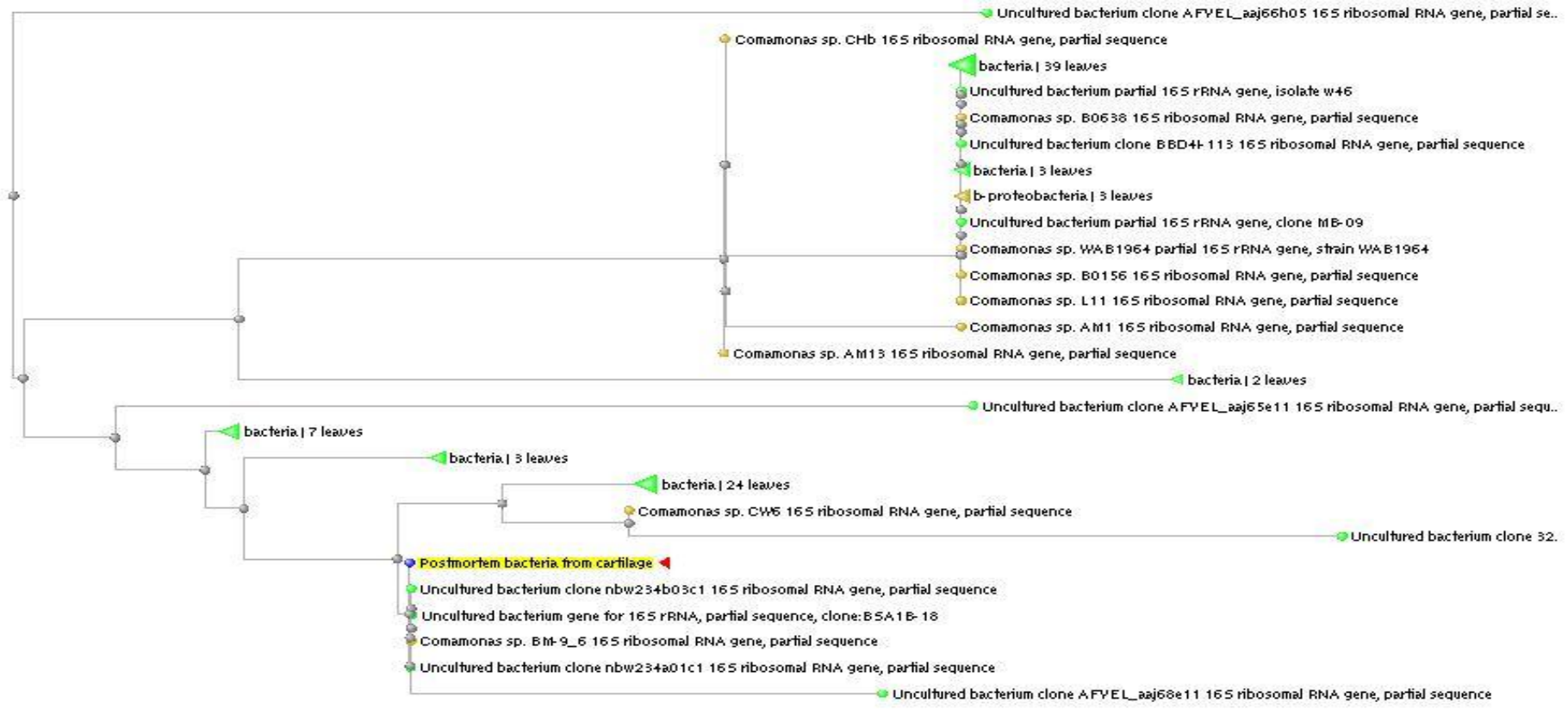


Figure 3.37: Phylogram exhibiting the relationship of the postmortem bacteria to other sequenced species.

It can be seen from figure 3.37 that the crystal forming bacteria was most closely related to a *Comamonas* species and a number of unidentified cultured bacterium. Hence, the cultured species could be a *Comamonas* sp.

3.3.12 Additional animal models

Cow

Eight cow hooves (2 interred in each grave) were interred in shallow graves and excavated after, 14, 18, 21, 25 days of burial in March 2010. A similar decompositional process was observed, comparable to the porcine samples. Figure 3.38A shows the hoof ready for internment in a grave at Compton campus. Figure 3.38B shows the hoof after 25 days of burial.

Figure 3.38: Macroscopic appearance of a cow hoof. A: fresh, ready for interment, B: after 25 days of burial.

The cartilage was dissected from the hoof using a similar method utilised with the porcine samples. The collected cartilage was subjected to SEM analysis which revealed crystal formations similar in morphology to the crystals observable on the porcine samples. In figure 3.39, three large crystals are easily visible, but in addition there are numerous smaller crystals present, possibly indicating that crystals may form as small structures increasing in size over time. SEM-EDX was conducted on these crystals (table 3.7) to determine their elemental composition.

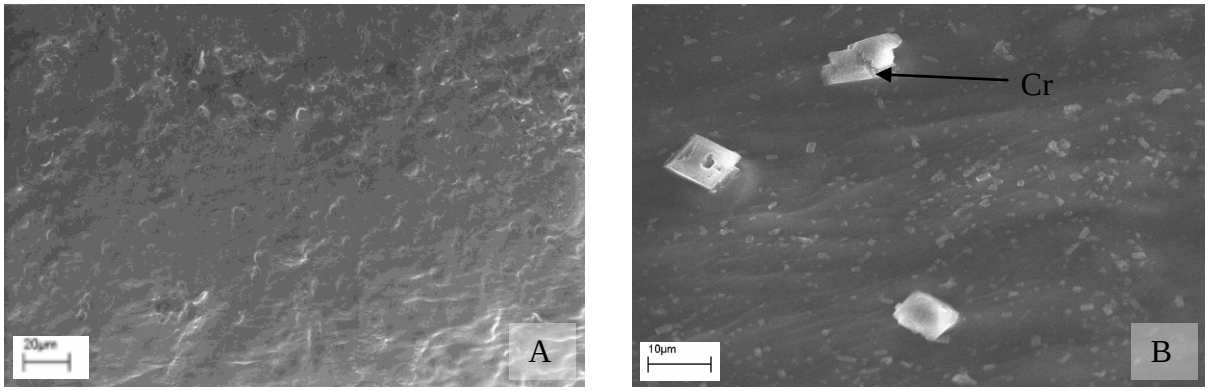


Figure 3.39: Crystal formation on the articular surface of cow cartilage. A: control, B: 25 day sample. Cr: crystals.

Table 3.7: The chemical composition of the crystals found on cow cartilage postmortem. Dominant elements are highlighted in bold.

Element	Weight %
C K	**36.21**
N K	**17.82**
O K	**41.10**
Na K	0.21
Mg K	**1.80**
P K	**2.18**
S K	0.26
Cl K	0.19
Ca K	0.24

Table 3.7 illustrates that the crystals forming on the surface of the cow cartilage have a similar chemical composition to the crystals found on porcine samples. The five dominant elements (C, N, O, Mg and P) were present. Additionally, the other elements found (Na, S, Cl and Ca) are also consistent.

Goat

Goat hooves were interred in similar conditions to the porcine and cow samples. Two trotters were interred in each grave and excavations occurred at seven, 10, 15, 20 and 23 days in March 2010.

Figure 3.40A illustrates the goat hoof prior to burial. These samples were significantly smaller than the porcine and cow samples, but followed a similar decompositional process, although the decomposition occurred markedly faster. External liquefaction in goat hooves was noted at 20 days of burial (figure 3.40B) whereas external liquefaction in porcine sample was noted after 32 days of burial.

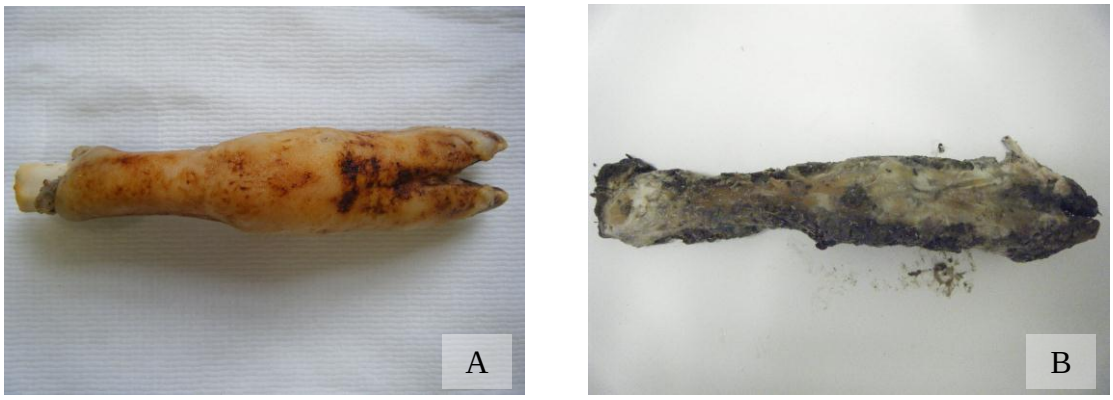


Figure 3.40: Macroscopic analysis of a goat hoof. A: prior to burial, and B: after 20 days of burial.

Cartilage was harvested by dissection and subjected to SEM and SEM-EDX analysis (figure 3.41 and Table 3.8). SEM analysis proved the presence of crystals on the surface. These were similar in morphology to the crystals seen in both the porcine and cow samples. In figure 3.41, large crystals were observed although these were low in abundance. However, numerous smaller crystals were seen forming on the sample. SEM-EDX showed that these crystals contained C, N, O, Mg, and P as the five most dominant elements concurring with the EDX results from both the porcine and cow samples (see table 3.8).

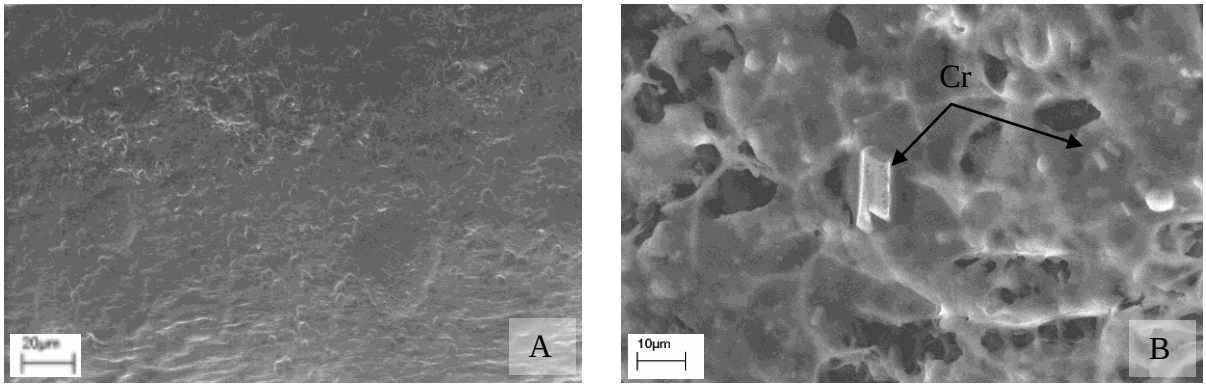


Figure 3.41: SEM micrograph illustrating the presence of crystals on goat cartilage postmortem. A: control, B: 20 days postmortem. Cr: crystals.

Table 3.8: The elemental composition of the crystals forming on goat cartilage postmortem. Dominant elements are highlighted in bold.

Element	Weight %
C K	**20.89**
N K	**4.14**
Na K	0.10
Mg K	**1.54**
P K	**1.92**
S K	0.04
Ca K	0.25
O K	**71.14**

Table 3.9 details the standard deviations and means for all of the SEM-EDX data gathered for the crystals obtained from the pig, cow and goat samples along with the crystals precipitated by the bacteria and finally the synthetic struvite. The five most abundant elements are detailed. The statistical data illustrates that while C, N, O, Mg and P are present within each of the crystal types, there is little similarity between each of the crystals.

Table 3.9: Statistical data (standard deviation and mean) for crystals collected from pig, cow and goat cartilage along crystals precipitated by bacteria and crystals of synthetic struvite. C: carbon, N: nitrogen, O: oxygen, Mg: magnesium and P: phosphorus.

Element	Statistical Analysis	Pig	Bacteria	Goat	Cow	Synthetic Struvite
C	**Mean**	39.87	14.75	19.83	34.49	30.55
	std	6.93	0.78	0.95	2.67	5.02
N	**Mean**	13.66	8.09	4.52	16.50	5.48
	std	2.08	0.39	0.30	1.36	0.80
O	**Mean**	40.11	58.47	24.93	43.02	42.12
	std	6.24	0.64	32.68	2.75	4.09
Mg	**Mean**	2.02	6.81	1.73	2.37	8.86
	std	1.16	0.13	0.22	0.60	0.69
P	**Mean**	2.27	8.93	2.56	2.78	12.29
	std	1.27	0.47	0.93	0.79	1.06

3.4 Discussion

Obvious ordered degradational changes (autolysis, putrefaction and skeletonisation) occurred to the trotters over time. Decompositional changes to cartilage were also documented as the postmortem interval increased. These changes included a change in colour from white to pink and finally to a dull cream, a change in surface texture and a loss of cartilage covering the articular facet. The successful application of the grading presented by Fuller *et al.,* (2001) (table 3.2) allowed for a score (from 0-4) to be attributed to the cartilage during postmortem analysis. H&E staining illustrated the loss of nuclear material over time with the greatest loss between six and nine weeks (figure 3.15). This is consistent with the findings of Lasczkowski *et al.,* (2002) who documented a loss of chondrocytes as the PMI increased. Unfortunately, the application of further histological stains such as Titan yellow yielded no positive or accurate data and are of limited value in this context (chapter 3.3.7). Little variation in decomposition was observed between the winter and summer. Cartilage became unrecognisable at 12 weeks postmortem at which point it was degraded to an extent at which it was of no value in this context.

Analysis by scanning electron microscopy (SEM) illustrated the presence of crystals on the cartilage surface at approximately three to six weeks postmortem (figure 3.16). Their presence is consistent in all eight replicates conducted, both in winter and summer. The largest crystals were approximately 10 µm in size, however significantly smaller crystals were also observed. This indicates that the crystals precipitate as small crystals and increase in size over time. Further experiments in the absence of soil showed that these crystals still form but within seven days (chapter 3.3.6). This indicates that their formation is not soil dependent. However, the data, for the speed of crystal formation in this context, cannot be compared to the data generated from samples interred in soil due to the differences in the interment environment.

It is hypothesised that the crystals on the cartilage surface disappear after six weeks due to exposure of the cartilage to acidic conditions within the grave environment enabled by the loss of surrounding tissue resultant from the decomposition process. This hypothesis is supported by the analysis of the effects of acidic and neutral solutions on the persistency of the crystals (chapter 3.3.10). However, this hypothesis requires further scrutiny.

SEM analysis showed that cocci bacteria were located in close proximity to the crystal structures (figure 3.17). Subsequent inoculation of cartilage on B-41 media resulted in the growth of bacteria, and after four days the precipitation of crystals with a similar, albeit larger, morphology to those found on the cartilage surface (chapter 3.3.4). GC-MS analysis,

conducted by a University in Poland, of bacterial fatty acids, presumptively identified three species: *Acinetobacter calcoaceticus, Acinetobacter iwoffii* and *Grimontia hollisae*. These species have not been previously reported on cartilage.

DNA analysis of the probable crystal producing bacteria resulted in identification as a *Comamonas* species. Unfortunately, the specific species could not be determined based on this analysis. *Comamonas* is documented to be ubiquitous in soil and water (von Graevenitz, 1985 and Jin *et al.,* 2009). Analysis via BLAST highlighted that the species cultured from the cartilage matched an unidentified cultured bacteria from human skin (Grice *et al.,* 2009), and the mammal gut (Ley *et al.,* 2008). *Comamonas* has been linked as an opportunistic human pathogen documented in cases of cystic fibrosis (Coenye *et al.,* 2002), endocarditis (Horowitz *et al.,* 1990), post-operative endophthalmitis (Reddy *et al.,* 2009), meningitis (Jin *et al.,* 2008) and as a result of a large feline bite (Isotalo *et al.,* 2000). *Comamonas* nucleic acids have been found in synovial fluid from reactive and postinfectious arthritis patients (Cox *et al.,* 2003). Cox *et al.,* (2003), hypothesise that the many bacteria encountered during their study may have been engulfed by macrophages and transported to inflamed joints. This study marks the first time *Acinetobacter calcoaceticus, Acinetobacter iwoffii, Grimontia hollisae* and *Comamonas* sp. have been documented on cartilage postmortem.

Scanning electron microscopy coupled with energy dispersive x-ray analysis (chapter 3.3.5) of the crystals from the cartilage surface and bacterial colonies established that carbon, nitrogen, oxygen, magnesium and phosphorus were the five most dominant elements. Although the morphology was similar between the crystals their size was different. The crystals on the cartilage surface were, on average, between 5 and 10 µm whilst the crystals precipitated from the bacteria were several millimetres in size. This variation in size between the samples could be explained due to the precipitation of bacterial crystals on a specialised media rich in magnesium and phosphorus and other relevant elements. Beavon and Heatley (1962) documented larger crystal formation with an increase in magnesium concentration to the culture media. Conversely the crystals on the cartilage surface are formed on a finite substrate, significantly smaller and with lower concentrations of the aforementioned elements. Furthermore, this cartilage substrate was decomposing leading to a loss of the substrate resulting in the formation of smaller crystals.

The utilisation of SEM and SEM-EDX of the cartilage surface allowed for easy identification of the crystals (based on morphology and chemical composition) and differentiation of the crystals from non-relevant artefacts which can easily contaminate the samples.

A pH analysis of the cartilage surface revealed a shift in pH from 7-8 at around 22-25 days postmortem (chapter 3.3.9). This shift in pH correlated with the precipitation of the crystals on the cartilage surface (table 3.6) and statistical analysis illustrated this relationship to be highly significant ($P<0.0001$). Changes in the soil pH due to the presence of a decomposing carcass have been documented in the literature (e.g. Reed, 1958). The result is a shift in pH to an alkaline environment possibly due to the release of ammonia during the decomposition process (Hopkins *et al.,* 2000, Wilson *et al.,* 2007) as a result of deamination of amino acids (Forbes, 2008c). Similar reactions could be hypothesised regarding the pH shift on the cartilage surface. If the pH change to the cartilage surface is found to be consistent after further research (see chapter 7 for further research) then this finding alone could offer a preliminary PMI estimation without the need for complex or expensive instrumentation. This would have particular preference at a crime scene or in a mortuary setting.

Synthetic struvite was obtained and an EDX analysis of this was compared with the results from the crystals produced by bacterial colonies inoculated from the cartilage, concluding in a match for the five most dominant elements, although the concentrations of these elements were seen to vary between the samples (Chapter 3.3.5).

Analysis of the EDX data and the X-ray diffraction analysis could suggest that the crystals formed on the cartilage surface and by the bacterial colonies were struvite ($(NH_4)MgPO_4.6H_2O$). However, the resultant XRD for the synthetic struvite and bacterially produced crystals do not completely match. This is likely due to a phenomenon known as preferred orientation (Grattan-Bellew, 1975, Scintag Inc., 1999). In this instance where the bacterial crystals (which were long and flat) are laying on their flat surface without all crystallographic plans subjected to the X-rays. This results in a non-random distribution of the crystal faces (Scintag Inc, 1999) meaning the crystals are laying in the same orientation. In addition, the crystals vary in their formation (one is synthetic and the other biologic) which may account for some of the dissimilarities.

Struvite is mineralised magnesium ammonium phosphate containing equal molar concentrations of magnesium and ammonium phosphate (Lee *et al.,* 2009) and can form renal calculi resultant of pathological processes (Yendt, 1970, Aage *et al.,* 1997, Grases *et al.,* 1998, Hall, 2002). The general formula for the formation of struvite is, (Doyle, Parsons, 2002):

$$Mg^{2+} + NH^{+}_{4} + PO_3^{4-} + 6H_2O \rightarrow MgNH_4PO_4.6(H_2O)$$

Struvite is precipitated by certain bacterial species (Scudder, 1928, Rivadeneyra *et al.,* 1992, Rivadeneyra *et al.,* 1993) under alkaline conditions (Lee *et al.,* 2009) and forms as characteristic orthorhombic crystals (Doyle and Parsons, 2002, Lee *et al.,* 2009) which are documented as being rare in nature (Rivadeneyra *et al.,* 1993). However, struvite is known to form at sites where organic matter decomposes, (Rivadeneyra *et al.,* 1993) such as old graveyards (Rivadeneyra *et al.,* 1992). Struvite is reported to be sparingly soluble under neutral and alkaline conditions but readily soluble in acids (Chirmuley, 1994). The formation and precipitation of struvite by bacteria varies with changes in the physical and chemical properties of the environment (Rivadeneyra *et al.,* 1992). These publications provide evidence, (namely, bacterial involvement in the crystal precipitation, crystal formation under alkaline conditions, crystals of an orthorhombic morphology, and formation at sites of decomposition), to support the findings uncovered during this analysis.

The origin of the observed bacteria has yet to be determined. It has been concluded that the control cartilage does not produce bacteria when inoculated on a medium nor are bacteria observable by SEM analysis. Migration of the bacteria from the soil into the joint spaces has been eliminated by successful crystal precipitation outside of a soil environment. It is likely the bacteria originate endogenously or exogenously to the cadaver. The bacteria could be normally compartmentalised within the body during life, but as with putrefactive bacteria they could proliferate postmortem (Pounder, 1995, Dent *et al.*, 2004) and migrate through the synovial capsule and membrane onto the cartilage. The *Comamonas* sp. has been documented endogenously in the mammal gut (Ley *et al.,* 2008). Furthermore, an unidentified cultured bacterium matching the postmortem cartilage bacteria (highlighted through BLAST) was documented exogenously on human skin (Grice *et al.,* 2009). Further research is needed to focus on provenancing the bacteria. Analysis of other regions of the body for example, blood vessels, could highlight the presence of these bacteria and provide further information as to any migratory patterns. Furthermore, this analytical approach may highlight other areas where crystal precipitation could occur and depending on the chemical composition (e.g. an earlier pH change) of any such tissues could be used to determine a more specific postmortem interval estimate.

Application of these analytical methodologies (namely, SEM and SEM-EDX) to other animal models (chapter 3.3.12) produced successful results, with similarities between the crystal morphology and their chemical composition (C, N, O, Mg and P being the dominant elements), concluding that the formation of postmortem crystals is not limited to porcine

samples. Unfortunately due to time constraints, this research could only detail that the crystals occurred in other animal models but the rate of the onset of the crystals still needs to be clarified. Statistical analysis (std and mean) of SEM-EDX data of the crystal types (crystals collected from pig, cow and goat cartilage, crystals precipitated from bacteria and finally, crystals of struvite) illustrated that while C, N, O, Mg and P were the most abundant elements, they were not similar in relation to their quantitative values. The differences between the animal (pig, cow and goat) crystals could be due to the differences in the species of animal or the elemental composition of the cartilage itself. Furthermore, the difference between the bacterial crystal compositions compared to the others could be due to how the bacteria precipitate the crystals. The differences between the synthetic struvite compared to the other crystals could be due to the crystals being manmade as opposed to biological in origin.

One possible problem with this study could be that the site of amputation acted as a site of access for microfauna, in essence speeding up the decomposition process (e.g. Campobasso *et al.*, 2001). This was however unavoidable as porcine trotters were cheap, abundant and easily obtainable making them desirable for this research.

Scudder (1928), concluded her paper explaining that the significance of ammonium magnesium phosphate precipitation by bacteria seems unimportant at the present time (1929). However, now, 82 years later, the production of this mineral by bacteria could help in pinpointing the time of death.

The examination of cartilage postmortem has provided valuable information to recommend that this method become a viable preliminary technique for determining the postmortem interval. The method could be used in conjunction with other methods such as forensic entomology (Goff and Flynn, 1991, Byrd and Castner, 2001). However, there is a dearth of methods available for determining PMI between a few days and several months especially in buried environments. Utilisation of this method may help narrow down the PMI to a range of zero to 12 weeks with techniques employed by most forensic services (e.g. SEM and SEM-EDX). There are limitations to the application of this data (as with all taphonomic experiments) namely environmental influences, such as temperature and soil type, which can result in different decomposition rates (e.g. Sledzik, 1998, Knight, 2004). This means that the data presented here may not be directly applicable to other geographical locations with different soil types or climatic conditions. It is also important to apply this method to human remains to see if the same processes occur. If so, then results from this research can be of use to forensic pathologists and anthropologists who routinely analyse remains in various stages

of decomposition. Furthermore, successful application of this method to other animal species may be beneficial to determine PMI in wildlife forensic cases e.g. poaching.

Chapter 4: Hair

"I have seen a thousand graves opened, and always perceived that whatever was gone, the teeth and the hair remained of those who had died with them. Is that not odd? They go the very first things in youth and yet last the longest in the dust".
(Lord Byron, 1820, letter to the publisher John Murray)

4.1 Hair Structure and degradation

Hair is comprised of dead cells which are bound together by extracellular proteins (Tortora and Grabowski, 2000, p.155). It has important biological functions, including protection of underlying tissues, insulation, for sexual display and to carry scent as a sensory organ (Harding, 1999, Wilson and Gilbert 2007). It is also reported to help camouflage animals against predators and act as filtering structures within the nasal orifice (Harding, 1999). Hair holds a variety of information about prior drug use, location history via isotope analysis and can contain important genetic information (Baumgartner *et al.,* 1989, Wilson *et al.,* 1995, Fraser *et al.,* 2006). Humans have three types of hair, 1) lanugo which is shed after 30 weeks in *utero*, 2) terminal hair which includes axial hair, beards, eyelashes, eyebrows, nasal hair, pubic hair and scalp hair, and, 3) vellus hair which is the un-pigmented downy hair that covers the body (Wilson *et al.*, 2007). The human scalp contains approximately 100,000 hair follicles, a higher density than other primates (Wilson *et al.,* 2007).

Cuticle cells make up the outside of the hair shaft. These hydrophobic cells are responsible for protecting the hair and providing the hair with its optical characteristics (Wilson *et al.*, 2007). Beneath the cuticle layer lays the cortex, which provides strength and flexibility to the hair (Wilson *et al.,* 2007). Centrally, within the middle of the hair shaft, lies the medulla, however, this is not consistently present in all hairs. It can be continuous or discontinuous and is filled with fluid or air (Wilson *et al.*, 2007).

Growth of hair occurs via mitosis at the hair follicle (Harding, 1999, Wilson *et al.*, 2007). The hair follicle is made of an outer and inner sheath (Tortora and Grabowski, 2000, p.149, Tortora and Derrickson, 2009, p.155). Situated at the base of the follicle is the bulb of the hair which contains the papillia and allows for infiltration of blood vessels. The bulb also houses the matrix which is a layer of germinal cells (Tortora and Derrickson, 2009, p.155). Hair follicles go through a growth cycle with each stage lasting for different time periods as detailed in figure 4.1.

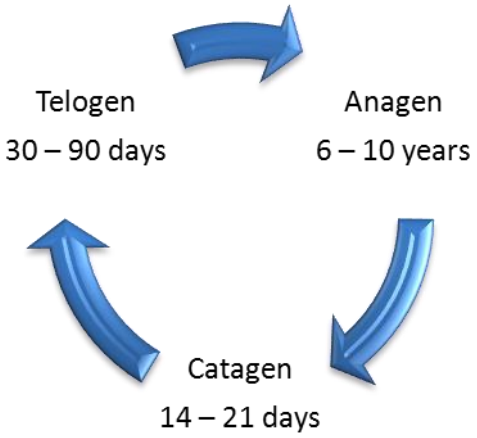


Figure 4.1: Hair follicle cycle. The times for each stage of the cycle are taken from Wilson et al., (2007).

During the Anagen phase cells within the hair matrix divide. New cellular material is attached to the base of the hair root which is pushed upwards resulting in hair lengthening. During this process, the cells become keratinised and die (Tortora and Derrickson, 2009, p.157). In the Catagen (regression) stage, the division of cellular material within the hair matrix ceases and the hair follicle atrophies (Tortora and Derrickson, 2009, p.157). After the regression stage, the hair enters the Telogen (resting) phase, this is where the hair is mature (Wilson *et al.*, 2007) and will eventually fall from the follicle, at which point the follicle growth cycle starts again.

The shaft of the hair is naturally covered in oils secreted from various sweat glands. For scalp hair this oil is produced by sebaceous and eccrine glands (Wilson *et al.*, 2007).

Under favourable conditions hair can persist for hundreds of years (for examples see the mummified remains presented by Musshoff *et al.*, (2009)).

Postmortem, roots of hairs in the anagen phase will undergo decompositional changes, the primary change is a black band forming approximately 0.5 mm above the bottom of the root. When viewed via microscopy these are actually air filled spaces (Tafaro, 2000, Linch and Prahlow, 2001). Changes also occur in the form of a brush like formation to the hair root after it has been pulled from a follicle (Tafaro, 2000). These changes, offer no information as to the time since death but can help in determining if a hair was pulled out ante or postmortem (Tafaro, 2000, Linch and Prahlow, 2001). Chang *et al.*, (2005) detail microscopic changes to hair over a period of 25 years. They discuss pores progressing into the medulla and loss of the cuticle layer. However, this was not consistent with some hairs retaining their cuticle layer.

Ultimately the researchers conclude that the time taken for these aforementioned changes is variable. The results of this study offer no information applicable to PMI estimation in a forensic context. Wilson *et al.,* (2001) explain the interaction of hair with the depositional environment and document the breakdown of cortical cell boundaries, infiltration of material from the burial matrix (soil), alteration of proteins and the loss of S-S cysteine links.

Hair that is in contact with soil has been noted to attract fungi over time. Griffin, (1959, 1960) showed a succession of characteristic fungal growth on hair: the first colonisers are highly competitive saprophytic fungi for example, *Fusarium* sp., some species of *Penicillium* and members of the *Muscorales* (Griffin, 1960). These are followed (with some possible overlap) by *Chaetomium cochlioides, Gliocladium roseum, Humicola* sp. and other *Penicillia*. The final stage (those fungi that can utilise the resilient components of hair) can include *Keratinomyces ajelloi* and *Microsporum gypseum*. The fungi sporulate at peaks of activity and these spores will survive in dormancy (Griffin, 1960). English (1965) presents information on how fungi invade keratinised tissue using fronded mycelium and boring hyphae along with the effects of such activity on the hair itself.

Keratinolytic fungi are found in the soil and are responsible for the breakdown of other keratinised and non-keratinised materials such as, hides, furs, claws, nails, and horns (Sharma, Rajak, 2003). They are able to exploit proteins containing cystine as a sole source of sulphur, carbon and nitrogen (Kunert, 1988, 2000). Fungi are known to degrade such materials via mechanical and enzymatic digestion (Wilson *et al.,* 2001b) and while the external appearance of the hair may seem intact, the inner aspects may be heavily degraded (Wilson *et al.,* 2001b). Fungal keratinolysis involves proteolysis and denaturation of the substrate via the cleavage of disulphide bridges by excreted sulphite, allowing for the hydrolysis of keratin by fungal proteases (Wilson, 2008). The fungi alkalinise their immediate environment via deamination and the production of ammonia (Kunert, 2000). However, a decrease in pH may be observed due to the neutralisation of ammonia via production of inorganic sulphate, S-sulphocysteine and thiosulphate from the oxidation of sulphur (Kunert, 1988). When exposed to a keratin containing food source fungi asexually multiply and produce conidia. Similarly to Griffin (1960), Sharma and Rajak (2003), detailed a succession of keratinolytic fungi on a suitable substrate; in the first instance non-specialist fungi will breakdown the non-keratinised portions. This is then followed by colonisation of true keratinophilic fungi.

The phenomenon of fungal degradation of hair has attracted the attention of the forensic community (DeGaetano *et al.,* 1992, Linch and Prahlow, 2001). The effects of putrefaction on

remains in a burial will affect the microenvironment and can limit the effect of keratinolytic fungi (Wilson, 2008). These factors are poorly understood and may result in a negative impact on PMI estimates.

The current research focused on the degradation of hair in a burial environment is of interest but there is still a lack of understanding regarding the effects of the burial environment (pH, elemental composition, moisture content, aeration etc.) and even cosmetic processes on the hair itself. The effects of such phenomena could have a detrimental effect on the hair thus allowing fungi to exploit any developed weaknesses.

4.2 Methods

4.2.1 Hair procurement

All hair utilised during the experiments was human in origin. For the first experiments hair from numerous and anonymous donors (including the principal researcher) was collected from 'the Basement' barbers located at 33, Lichfield Street, Wolverhampton.
However, only hair from the principal researcher was utilised for the second, third and fourth experiment for comparison purposes. All of the hair utilised was buried without sample pre-treatment (see chapter 4.2.2).

4.2.2 Burial set ups

In the first sample set, hair was buried to a depth of 20 cm at Compton campus (chapter 2). Burial occurred directly into the ground. The hair was tied to orange garden twine which was in turn, tied to the fencing of the enclosure. This allowed for easy location and recovery of the samples. However, despite the enclosure, scavenging activity resulted in the loss of some samples.
As a result of the scavenging activity, the second, third and fourth sample sets occurred on the roof of MA block of the University of Wolverhampton's city campus (figure 2.2). Soil was transported back from Compton campus and placed into terracotta pots with a 25 cm diameter. This was to ensure the continuity of the method. These samples were buried to a depth of 15 cm and a wooden stake was used to mark the exact location of the sample within the pot.
With all of the burial experiments, one sample set of hair was buried with close association to meat, whilst the other sample set was buried without. The meat was raw unsalted pork chop (3 cm x 3 cm blocks) and was utilised as an analogue for decomposing human remains. Table 4.1 details the burial intervals of each sample set.
Disinterment of the samples followed the standard archaeological protocol of removing small layers of soil at any one time (Hunter and Dockrill, 1996), to ensure a maximum recovery of the sample.
After excavation, the samples were stored in a freezer at -10°C until analysis. Initial analysis involved standard light microscopy using a Nikon 'Eclipse' ME600 (Nikon Corporation,

Tokyo, Japan). Three hair strands were analysed per sample set to ensure reproducibility of all results.

Table 4.1: Time intervals (weeks) for the burial of hair samples. Each sample set consists of samples buried with and without exposure to meat.

Burial interval (W: weeks)							
Spring and Summer				Autumn and Winter			
Sample set 1		Sample set 2		Sample set 3		Sample set 4	
Interval	Date	Interval	Date	Interval	Date	Interval	Date
3	22.04.08	5	02.07.08	5	13.10.08	4	07.11.08
9	03.06.08	10	06.08.08	10	18.12.08	8	05.12.08
14	08.07.08	15	10.09.08	15	22.01.09	12	04.01.09
19	12.08.08			20	26.02.09	16	30.01.09
24	16.09.08			25	02.04.09	20	27.02.09
						26	10.04.09

4.2.3 Lactophenol Cotton Blue Staining

Previously buried hair was placed into a Petri dish and covered in a premade solution of lactophenol cotton blue (LPCB) and left for five minutes to ensure uptake of the stain. The hair was transferred to a standard microscope slide and covered in a Canada balsam substitute (Brunel Micro LTD) and sealed with a standard microscope cover slip. Lactophenol blue kills the organism due to its phenol concentration but is ultimately responsible for staining chitin in fungal cell walls (Leck, 1999). Canadia balsam substitute is widely used due to its optical and refractive properties.

4.2.4 Light Microscopy

Light microscopy on the LPCB stained samples was conducted with an Eclipse E600 (Nikon) with a camera attachment. Magnification was at 100x or 400x. Images were not modified with any imaging software. The entire length of the hair sample was analysed.

4.2.5 Scanning Electron Microscopy

Hair samples were adhered to microscopy stubs via carbon adhesive pads and placed directly into the scanning electron microscope. On occasion, some samples were gold coated to obtain a sharper image. Analysis was conducted with an EVO 50 Scanning Electron Microscope (Zeiss). The magnification was not consistent and varied depending on what was observed. The entire length of the hair sample was analysed.

4.2.6 Culturing of fungi

Collected hair samples were inoculated on to a malt extract agar (MEA: 3 g Agar 2, 4 g ME in 200 ml distilled water) with a sterile metal needle. The MEA was treated with an antibiotic (Ampicillin: 0.10 g in 20 ml distilled water) to retard bacterial growth. The inoculated fungal samples were placed in an incubator at 25˚C for between five and seven days to allow for substantial development. Sub culturing of the fungi occurred several times to ensure pure cultures were obtained. Pure cultures of fungi were stored in glycerine at -80°C as a fungal stock.

Prior to DNA analysis, the pure fungal cultures were inoculated in liquid malt extract (ME: 2 g malt extract per 100 ml distilled water) and incubated in an orbital shaker at 30˚C overnight which resulted in prolific growth of fungal structures. The fungi were harvested in filter paper and pressed firmly to remove any superfluous ME, the samples were then wrapped in aluminium foil and immediately stored at -20˚C.

4.2.7 Growth of fungi on hair placed on a water agar medium

Freshly cut hair was inoculated with the pure fungal colonies obtained in the previous experiment (4.2.6). The hair utilised was from one source (the principal researcher). The inoculated hair was placed on a water agar medium (2 g agar 2 / 200 ml of distilled water) and left at room temperature. This analysis focused on identifying any hair damage caused by fungal species outside of a soil (burial) environment. This analysis eliminated degradation caused by insects and adverse soil conditions.

Initially samples were collected every week for five weeks, and then this was altered to every two weeks, then every four weeks up until a total of 24 weeks had elapsed. Samples were collected using aseptic techniques to minimise cross contamination.

4.2.8 Fungal DNA extraction

For the DNA extraction a revised method of Raeder and Broda (Raeder and Broda, 1985) was utilised. Collected fungal samples were placed into a sterile mortar with liquid nitrogen and ground to a fine powder with a sterile pestle. Equal quantities of phenol:chloroform (Fisher scientific) and extraction buffer (extraction buffer per 100 ml: 200 mM tris-HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS), to a maximum of 4 ml, was added and ground with the sample until a slurry remained. This slurry was collected into sterile 2 ml microcentrifuge tubes (Sarstedt) and centrifuged at 13,000 rpm for 30 minutes. The supernatant was transferred into a new tube along with 1 µl of RNAse A (Sigma) and left at room temperature for 30 minutes. 700 µl of phenol:chloroform was added and the tube was inverted several times and centrifuged at 13, 000 rpm for two minutes to remove any cell components or RNA which may impede further analysis. The supernatant was transferred to a microcentrifuge tube and 1 ml of chloroform was added to remove any excess phenol which may interfere with polymerase chain reaction (PCR). This solution was centrifuged at 13.000 rpm for two minutes. The supernatant was transferred into a new tube along with 30 µl of sodium acetate (3 M, pH 5.2) and 1 ml of ice cold 95% ethanol, the mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the pellet contained at the bottom of the tube was allowed to dry for 30 minutes at room temperature. 100 µl of distilled water was then added to re-suspend the pellet and the sample was then frozen at -20˚C.

4.2.9 Fungal DNA quantification

DNA samples were thawed and homogenised by gentle tapping of the microcentrifuge tube. To make a 100 x dilution, 890 µl of TE buffer and 9 µl of DNA extract were transferred to a quartz cuvette and placed into a UV spectrophotometer (Helios α, Thermo) set to measure the absorbance of light at 260 nm. TE was utilised as a blank control.

4.2.10 Gel Electrophoresis

Gel electrophoresis was conducted to test for the presence of DNA and determine if extraction was successful. A 2% agarose gel was utilised with, in the first instance, ethidium bromide (Ethidium bromide concentration: 25 µl in a 50 ml gel or 50µl in 100 ml gel). In the later DNA analysis ethidium bromide was substituted with WebGreen (15 µl, Web Scientific), which is a safer, non-carcinogenic alternative. The duration and voltage at which the electrophoresis was run was dependent on the circumstances in hand.

4.2.11 Polymerase Chain Reaction of extracted fungal DNA

PCR or Polymerase Chain Reaction is a process utilised to exponentially amplify a specific region of DNA *in vitro* (Mullis and Faloona, 1987). The DNA is denatured and target regions of DNA are amplified using primers (oligonucleotides) (Mullis and Faloona, 1987). Primers are designed to be complementary to the two 3' ends of the DNA segment to be amplified (Garrett and Grisham, 1999, p.417, McPherson and Geir Moller, 2006, p.26, Goodwin *et al.*, 2007, p.54). DNA polymerase (Taq) is added and the reaction occurs. Once a reaction is completed, the DNA is denatured and the process occurs again (Mullis and Faloona, 1987), allowing for the aforementioned exponential amplification. The PCR process occurs in a Thermocycler which allows for the desired times and temperatures to be programed for the initial denaturation, denaturation, annealing, extension and final extension parameters of the PCR cycle.

ITS (internal transcribed spacer) regions of fungal DNA were the focus of the PCR. These regions are highly variable and have been widely used to study relationships between fungal species (Viaud *et al.*, 2000). Fungal nuclear ribosomal DNA (rDNA) is comprised of three genes, a large subunit (26S), small subunit (18S) and the 5.8S gene (Atkins and Clark, 2004) which are highly conserved and not changed during mutation and cannot be used to distinguish between fungi at the species level (Moore *et al.*, 2002). Each of these rDNA components are separated by the ITS region (Atkins and Clark, 2004). Universal primers for the ITS regions are now commercially available and amplify the noncoding regions between rDNA genes (White *et al.*, 1990).

PCR amplification of the 5.8S ribosomal regions of fungal DNA was conducted using ITS1 and ITS4 primers (using a method adapted from White *et al.*, 1990). The PCR master mix

used was, per 100 ml: 8 µl dNTPs, 20 µl of 10x PCR buffer (Fermentas: 750 mM Tris HCl, 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20), 4 µl of ITS1 primer, 4 µl of ITS4 primer, 1µl of Taq polymerase, 20 µl of $MgCl_2$, 43 µl of sterile distilled water.

DNA (amount: 100ng / 50 µl reaction) was diluted using sterile distilled water to make up a 25 µl volume which was then mixed, in a 0.2 ml PCR tube (Fisher Scientific), with 25 µl of the PCR Master Mix to form a combined 50 µl reaction volume.

The PCR tubes were loaded into the PCR thermocycler (Uvigene) with the following cycle reactions: Initial denaturation at 95ºC for five min, 25 cycles of denaturation, annealing and extension, at 95ºC for 0.5 min, 58ºC for 0.5 min and 72ºC for 2 min respectively. This was followed by final extension at 72ºC for 10 min.

After the PCR reaction, 3µl of loading buffer was added to 15µl of PCR product and loaded into a 2% agarose gel. Hyperladder 1 (Bioline) was also loaded and utilised as a size marker. Hyperladder 1 is used as a size marker for samples between 200 and 10,000 base pairs.

4.2.12 Fungal DNA sequencing and bioinformatics

Prior to DNA sequencing, the PCR products were purified using ExoSAP-IT (Affymetrix) which removes unused primers and nucleotides. 4 µl of ExoSAP-IT was added to 10 µl of PCR product in a 0.2 ml PCR tube (Fisherbrand). The samples were incubated at 37ºC for 15 min to degrade the remaining primers and nucleotides. Then the samples were incubated at 80ºC for 15 min to deactivate the ExoSAP-IT.

After purification the samples were sent to the DNA sequencing and Services Unit at Dundee University for sequencing. Additionally, 15 µl of the ITS1 and ITS4 primers were also dispatched with the samples. The results were analysed using a bioinformatics software called BLAST to obtain an identification.

4.3 Results

The hair demonstrated gradual degradation over time with increasing colonisation by fungal species. The following section will detail the results for analysis of hair and fungal species using; lactophenol cotton blue, scanning electron microscopy, light microscopy, and finally, fungal species specification by DNA.

4.3.1 Lactophenol Cotton Blue of samples interred in terracotta pots

Figure 4.2 illustrates the degradation of human hair samples over 15 weeks. The samples were buried in Compton soil contained in terracotta pots. Figure 4.2A shows the control hair. No fungal structures were observable. Figure 4.2B and C show the five week samples with and without meat. The sample with meat lacks any observable fungal structures whereas the sample without meat has prominent fungal hyphae beginning to encase the hair shaft. Both the 10 week samples (figure 4.2D and E) show fungal growth. The sample buried with meat (figure 4.2D) has small fissures transversing the hair shaft. However, it is not possible to determine if these fissures are a result of mycological activity. The sample buried without meat (figure 4.2 E) was associated with numerous masses of circular fungal spores but the hair shaft was intact. Figure 4.2 F shows the 15 week sample buried with meat was associated with numerous circular fungal spores. The 15 week sample buried without meat was recovered but due to its fragility and reduced amount it was exclusively assessed by SEM analysis.

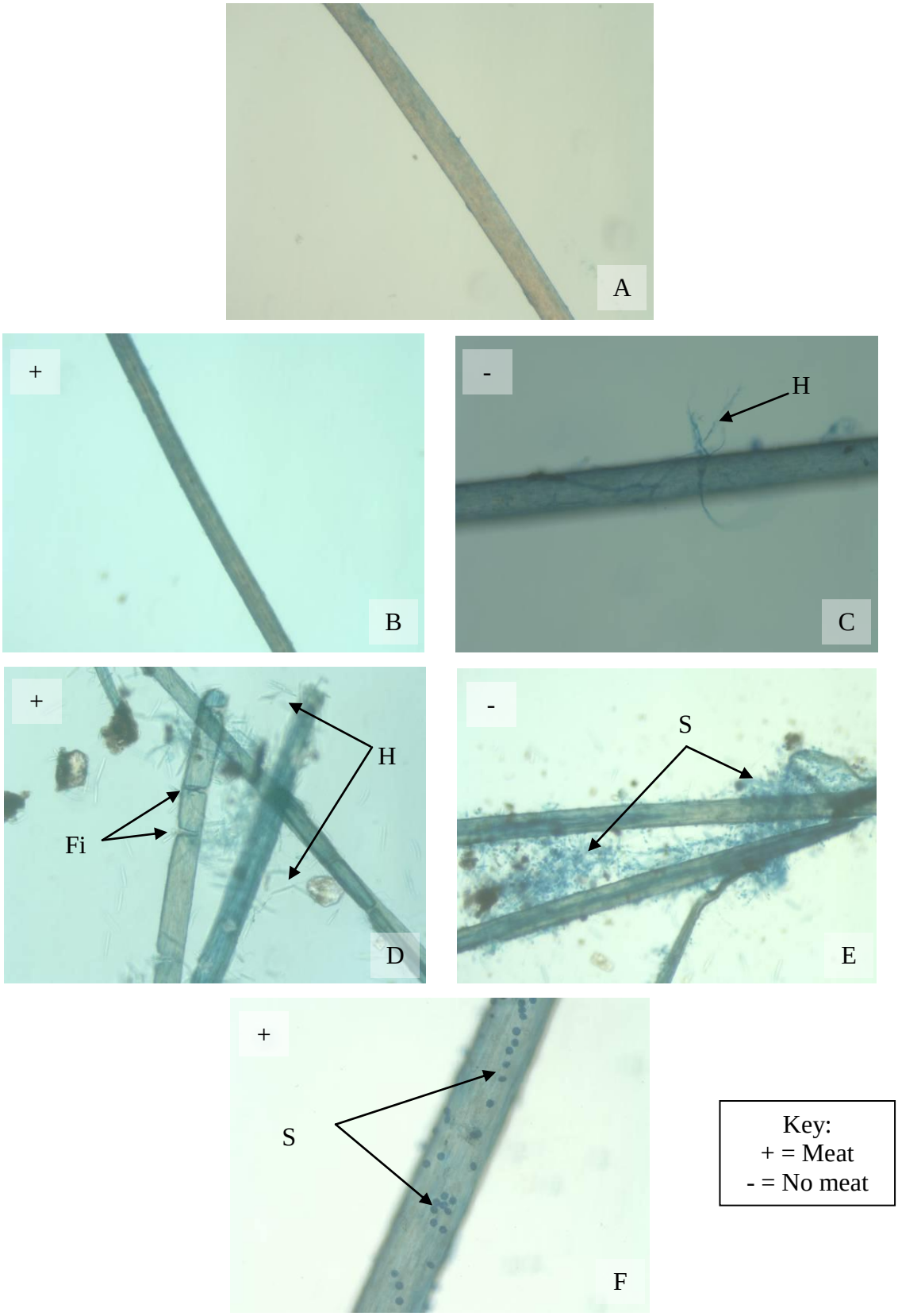
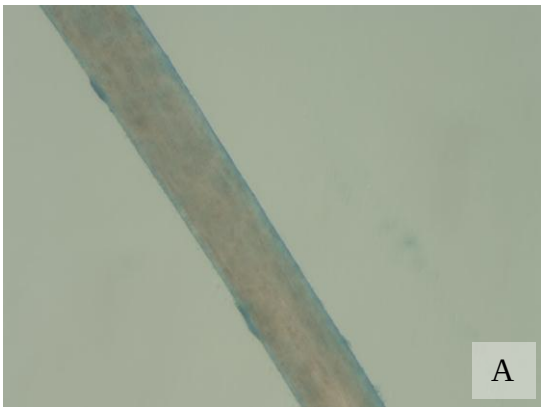


Figure 4.2: Degradational changes to hair interred in terracotta pots over 15 weeks. + (plus): with meat, - (minus): without meat. A: control hair, B and C: 5 week samples. D and E: 10 week samples, and, F: 15 week sample. H: hyphae, Fi: fissure, S: fungal spore.

4.3.2 Lactophenol cotton blue of samples interred directly into the ground at Compton campus

Figure 4.3 chronologically documents the degradation of hair over 24 weeks when interred directly into the ground. Figure 4.3A shows the control hair absent of any fungal structures. B and C show the samples at three weeks post burial. Fungal structures can be seen on the sample which was buried in association with meat (figure 4.3C). Fungal structures were visible only on the nine week sample buried without exposure to meat (figure 4.3E). The structures found on the nine week sample exposed to meat were likely to be artefacts of no relevance (figure 4.3D, based on morphology). At 14 weeks fungal structures were only visible on the sample buried without meat (figure 4.3G), there was no evidence of fungal activity on the sample buried in association with meat (Figure 4.3F). The 19 week sample buried with meat exhibited no fungal activity (Figure 4.3H) as opposed to the sample without any meat association (Figure 4.3I). Finally, the 24 week sample buried with meat showed some areas of blue staining. However, on closer scrutiny these areas had no thread like appearance and lack any clearly defined borders indicating these are small conglomerates of lactophenol cotton blue stain (figure 4.3J). Conversely, the sample buried without meat association had numerous small circular fungal spores (figure 4.3K).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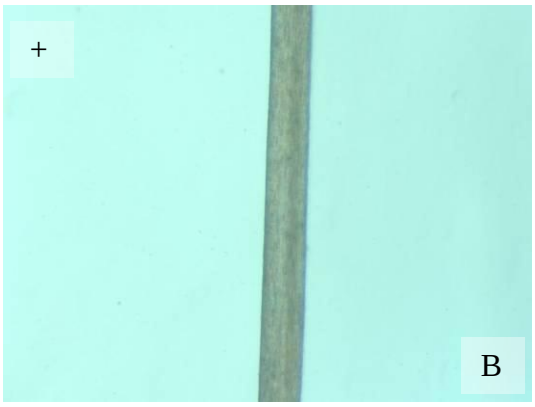
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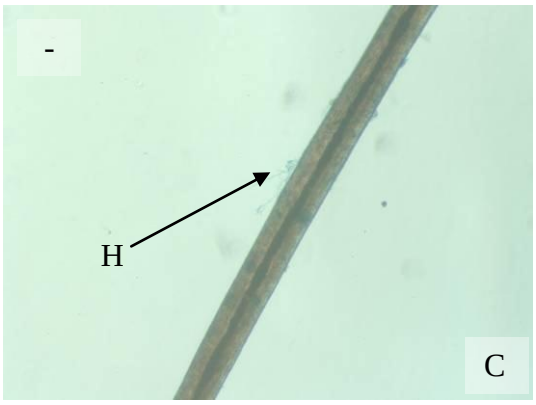
B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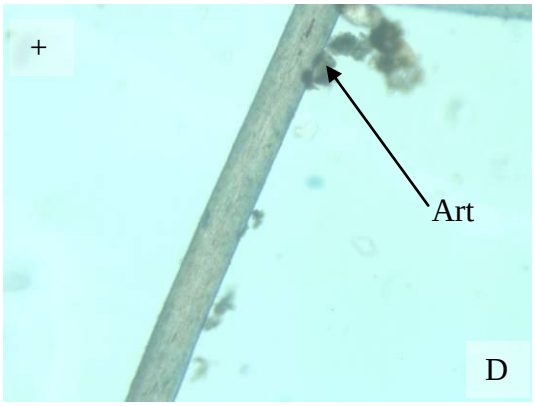
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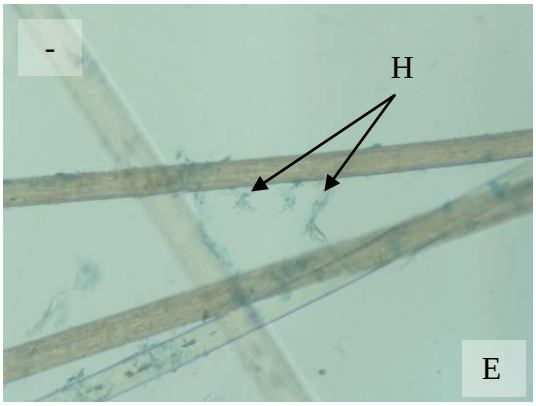
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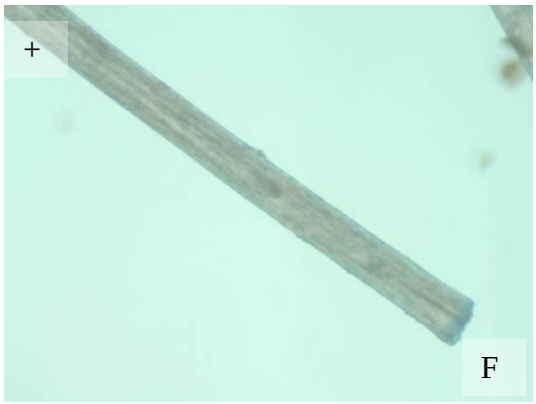
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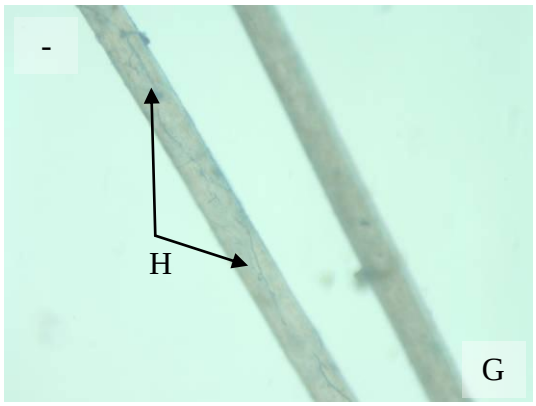
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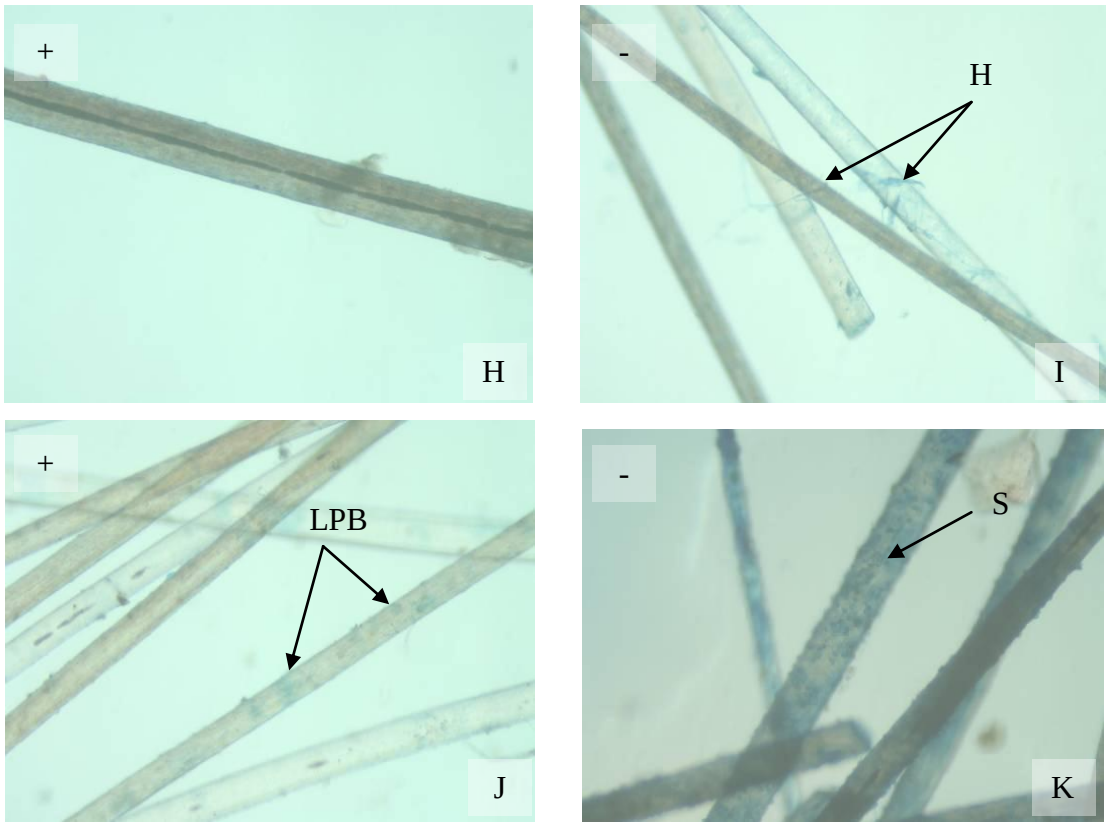

Figure 4.3: The degradation of hair when interred directly into the ground at Compton Campus over 24 weeks. A: control hair, B and C reflect 3 weeks, D and E: 9 weeks, F and G: 14 weeks, H and I: 19 weeks and J and K: 24 weeks. H: hyphae, Art: artefact, LPB: lactophenol blue, S: spore.

4.3.3 SEM analysis of hair buried at Compton without association with meat

Figure 4.4A illustrates the control hair. There were no fungal structures present, and the mounds present on the shaft were likely to be a cosmetic adhesive such as hair gel as the donor of the hair was known to use such products. The three week sample was structurally sound with the cuticle scales present but fungal structures were beginning to colonise the hair shaft (Figure 4.4B). The nine week sample was still intact with cuticle scales still present (figure 4.4C). There was a fibre like structure associated with the hair which was fungal in morphology. Soil debris was also present although of no value in this context. The fourteen week (Figure 4.4D) sample still possessed scales. Fungal structures including spores were easily recognisable. Some of the fungal hyphae appeared to be segmented. Soil debris was once again dominant but of no value. The nineteen week sample was surprisingly intact and in remarkable condition with the scales in good condition (Figure 4.4E). There was also

possible fungal hypha present. The twenty-four week sample had degraded (Figure 4.4F). There was an absence of cuticle scales. The middle section of the shaft exhibited signs of undulation which possibly reflects the cortex of the hair. Fungal structures were also present although minimal. The middle section of this sample exhibited two holes indicating substantial damage. The causative agent cannot be verified. It is possible that fungi could use this as a point of entry but this is just speculative.

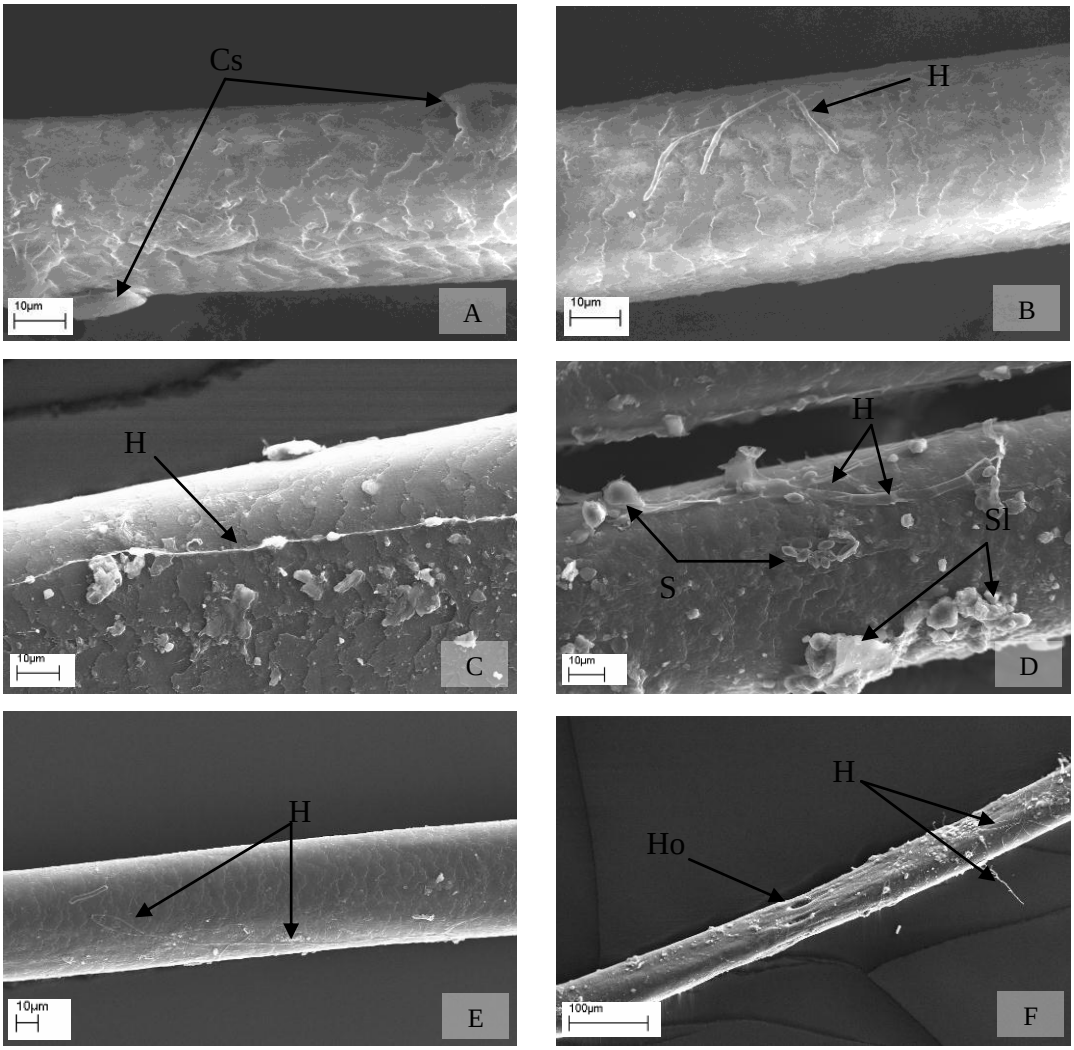

Figure 4.4: SEM analysis of the degradation of hair in the Compton soil at Compton without association with meat. A: control, B: three weeks, C: nine weeks, D: fourteen weeks, E: nineteen weeks, and F: 24 weeks. Cs: possible cosmetic, H: hyphae, S: spore, Sl: soil and Ho: hole.

4.3.4 SEM analysis of hair buried at Compton in association with meat

Figure 4.5A shows a control hair, which was in unremarkable in appearance. The three week sample was also unremarkable, lacking fungal structures but did have soil artefacts in association, which had no value in this context (Figure 4.5B). The nine week sample showed fungal growth in the form of hyphae (Figure 4.5C). Scales were still present with soil artefacts present. The fourteen week sample still retained cuticle scales and was structurally intact (Figure 4.5D). There were no identifiable fungal structures. The nineteen week sample was again intact structurally (Figure 4.5E). As with the fourteen week sample there were no identifiable fungal structures and soil artefacts were observed. The twenty four week sample was intact (Figure 4.5F) compared to the 24 weeks sample buried without meat (figure 4.4F). There was a thread like structure present this could be a fungal structure but this could not be substantiated based on this image.

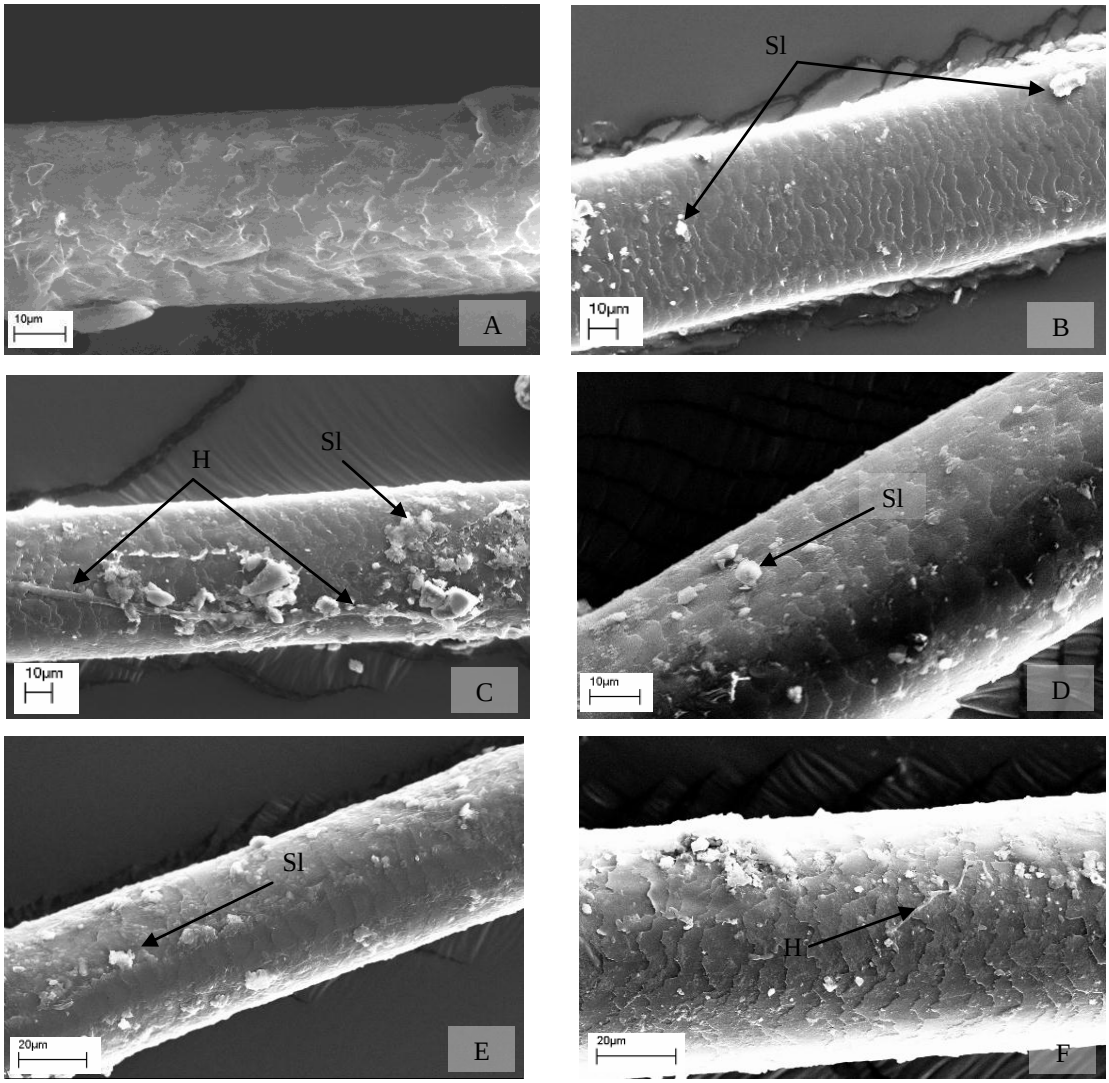

Figure 4.5: SEM analysis of the degradation of hair in the Compton soil at Compton with association of meat. A: control, B: three weeks, C: nine weeks, D: fourteen weeks, E: nineteen weeks, F: 24 weeks. Sl: soil and H: hyphae.

4.3.5 SEM analysis of hair interred in Terracotta pots without exposure to meat

Figure 4.6 illustrates the degradation of hair interred in terracotta pots without exposure to meat. The five week sample (Figure 4.6A) retained cuticle scales and fungal growth was observed in the form of hyphal structures represented on the entire sample. The ten week sample showed destruction of the cuticle layer with no remaining cuticle scales (Figure 4.6B). There was an abundance of fungal growth including the presence of numerous fungal spores. What remains of the hair shaft was undulated and could be representative of the bundles that make up the hair, exposed due to loss of the cuticle. The fifteen week sample had degraded to the extent that the shaft had broken down (Figure 4.6C). The cuticle was lost and sufficient

damage had been done to break the hair into its respective bundles. Hyphae were present on this sample.

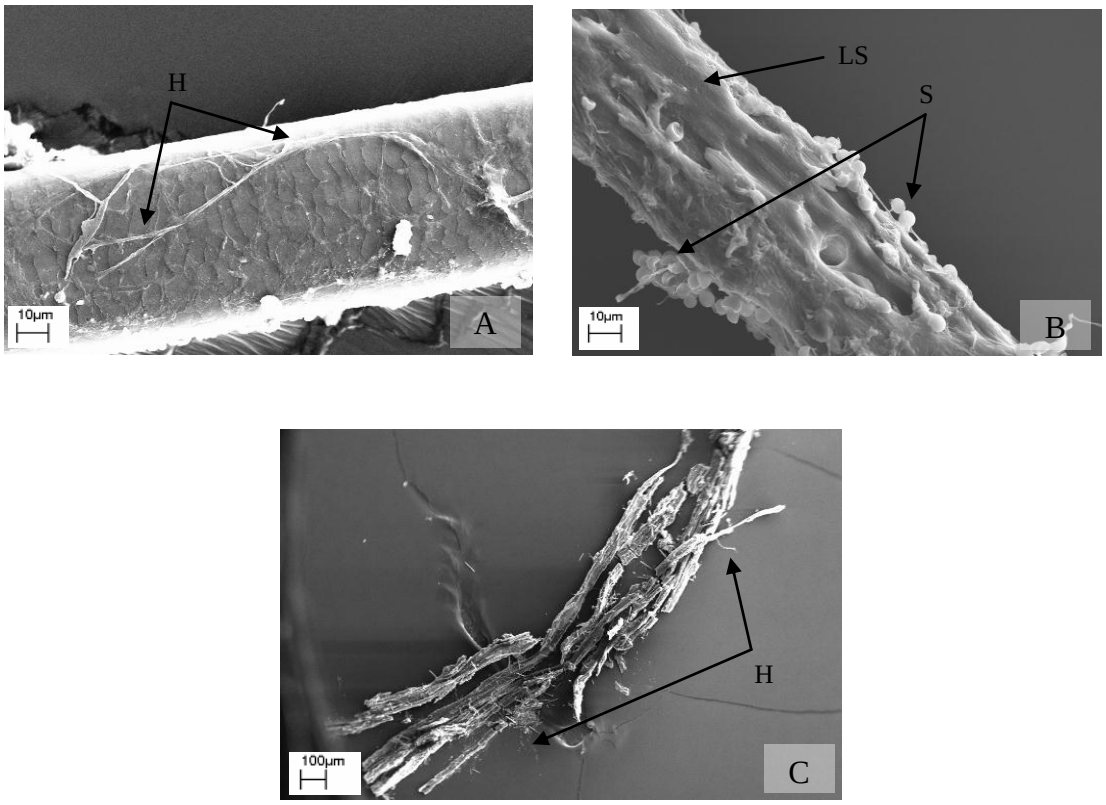

Figure 4.6: SEM analysis of hair samples without exposure to meat, interred in terracotta pots containing soil from Compton. A: five weeks, B: ten weeks, C fifteen weeks. H: hyphae, S: spores, LS: loss of cuticle scales.

4.3.6 SEM analysis of hair interred in terracotta pots with exposure to meat

Figure 4.7 illustrates only slight degradation of the hair shaft over time. The five week sample was visually intact with some fungal structures in association (Figure 4.7A). The scales were present and normal. The ten week sample demonstrates prolific fungal growth in the form of web like structures containing cylindrical fungal spores (Figure 4.7B). The cuticle scales were absent and some undulations are manifested. This could be due, again, to loss of the cuticle. The fifteen week sample, interestingly, still retained cuticle scales and had a reduced amount of fungal colonisation comparable to the ten week sample (Figure 4.7C). This could be due to variations in the burial environment. Soil artefacts were also in association but these had no evidential value in this context.

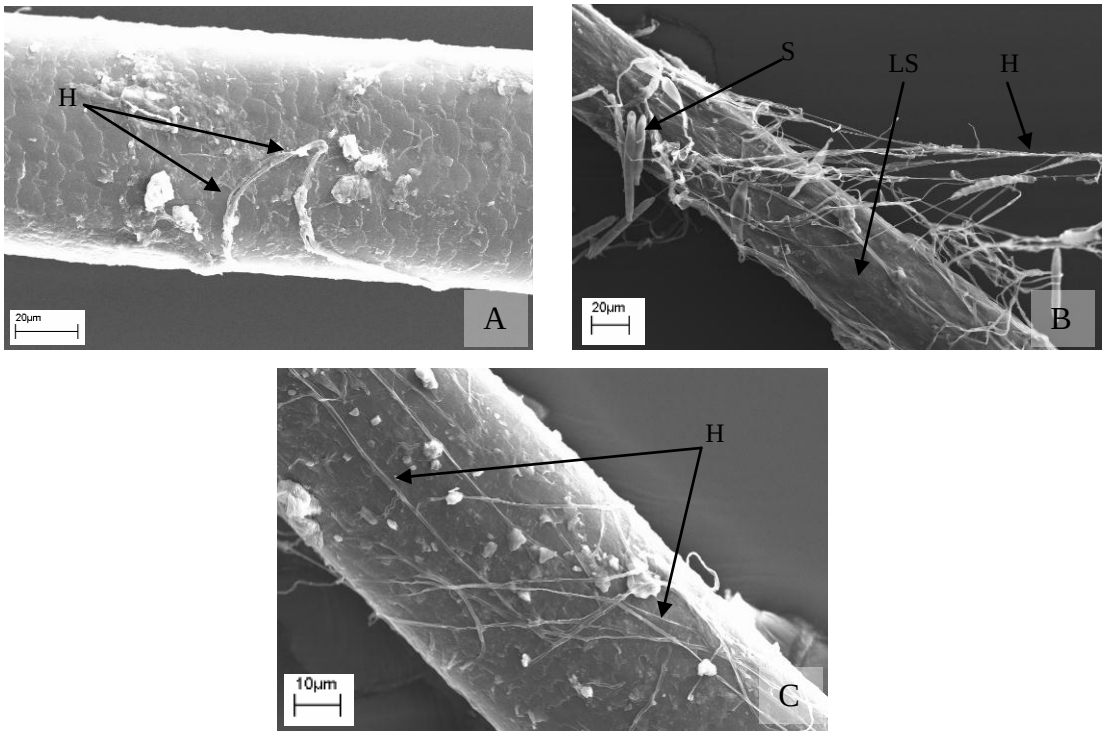

Figure 4.7: SEM analysis of hair samples, with exposure to meat, interred in terracotta pots containing soil from Compton. A: five weeks, B: ten weeks, C: fifteen weeks. H: hyphae, S: spores, LS: loss of cuticle scales.

4.3.7 Effect of fungi on hair – water agar experiment

This experiment observed the damage fungi caused to hair. The fungal species used were collected from the method detailed in chapter 4.2.6. Due to growth on a water agar adverse soil conditions and entomological degradation were eliminated. Due to the large number of micrographs taken, one sample (sample F) will be discussed in detail to illustrate fungal tunnelling to the hair shaft. The findings for the other fungal species will be detailed in table 4.2.

Figure 4.8 documents the tunnelling of hair by fungi. Fungal structures were not observed on these samples, but the presence of tunnels progressing from the cuticle layer through to the inner aspects of the hair shaft which was visible. Figure 4.8A shows the control hair with no tunnel structures. Tunnelling became obvious at three weeks incubation (figure 4.8B). At 24 weeks incubation the tunnelling was advanced (figure 4.8G), the tunnels became so dense that areas of the hair shaft were darker in appearance.

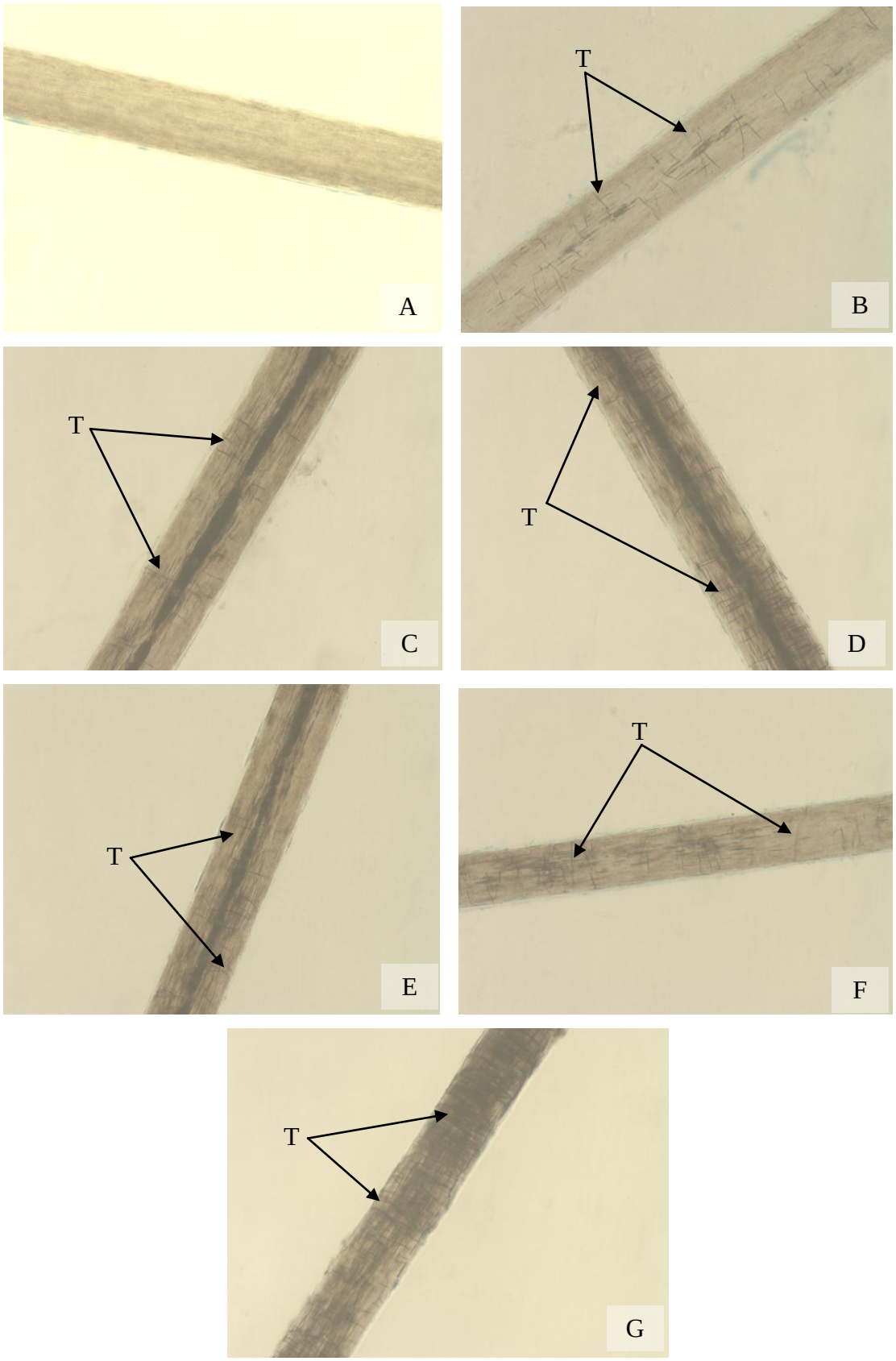

Figure 4.8: The gradual increase in tunnelling observed over time. A: control, B: three weeks, C: five weeks, D: nine weeks, E: thirteen weeks, F: fifteen weeks, G: twenty-four weeks. Light microscopy, 200x magnification. T: tunnelling.

Table 4.2 shows that fungal tunnelling was observed on 16 out of the 18 fungal samples. Sample f, (as shown in figure 4.8) presented with tunnelling in eight out of the 10 samples analysed, with the one and two week intervals absent of tunnelling. Two samples (b and o) exhibited no fungal tunnelling during the 24 week period. The majority of samples (a, c, d, e, g, h, i, j, k, l, m, n, p, q and r) only exhibited tunnelling in the later stages of the experiment. In contrast to these results, SEM analysis on all inoculated samples illustrated some degree of fungal colonisation to the hair shaft.

Table 4.2: Hair samples with tunnelling observable after inoculation with different fungal isolates. Isolate: the different fungal species, Time (weeks): the interval for sample collection, 0: the control, y: presence of tunnelling.

Isolate	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r
Time (Weeks)																		
0																		
1																		
2																		
3						y												
4						y												
5						y												
7						y	y										y	
9						y												
11						y							y				y	
13					y	y		y					y			y	y	
15	y		y			y	y	y	y	y	y		y				y	y
24	y			y		y	y		y	y	y	y	y	y			y	y

4.3.8 Morphology of damage to decomposing hair inoculated in water agar

Several manifestations of hair damage were encountered during the water agar experiment as shown is figure 4.9.

Fungi were responsible for the damage encountered during the water agar experiments. Figure 4.9A shows that the cuticle scales have been stripped away resulting in a hair shaft that was smooth in appearance. Furthermore, the hair shaft was uneven in morphology, possibly due to the removal of the cuticle of the hair. Figure 4.9B illustrates a prominent discontinuity (a hole) in the hair with fungal spores in close proximity, cuticle scales were still present. Figure 4.9C shows a transverse break in the hair shaft, where cuticle scales were absent.

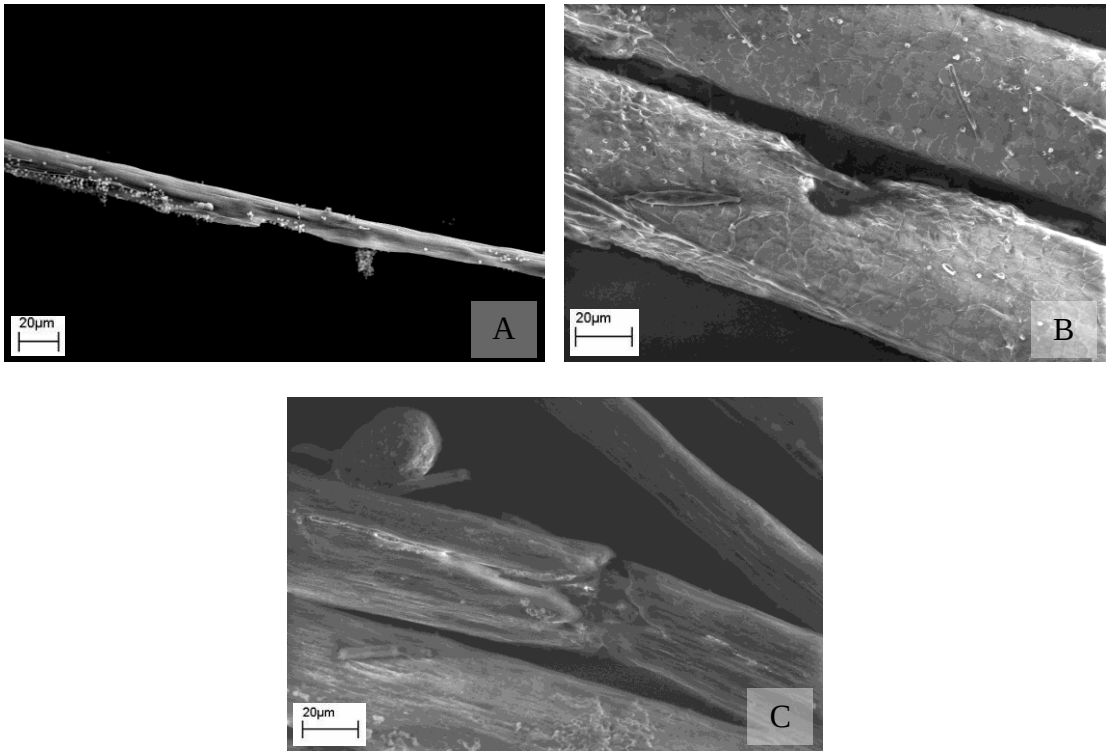

Figure 4.9: Damage to hair samples encountered during the water agar experiments.

4.3.9 Morphology of fungal spores observed during SEM analysis of decomposing hair

During SEM analysis of decomposing hair, four types of fungal spores were documented (figure 4.10). Figure 4.10A illustrates spherical spores with a irregular outer shell. Figure 4.10B shows oval spores, figure 4.10C shows spherical smooth spores and figure 4.10D shows tubular spores. All of these spores, probably originate from different species.

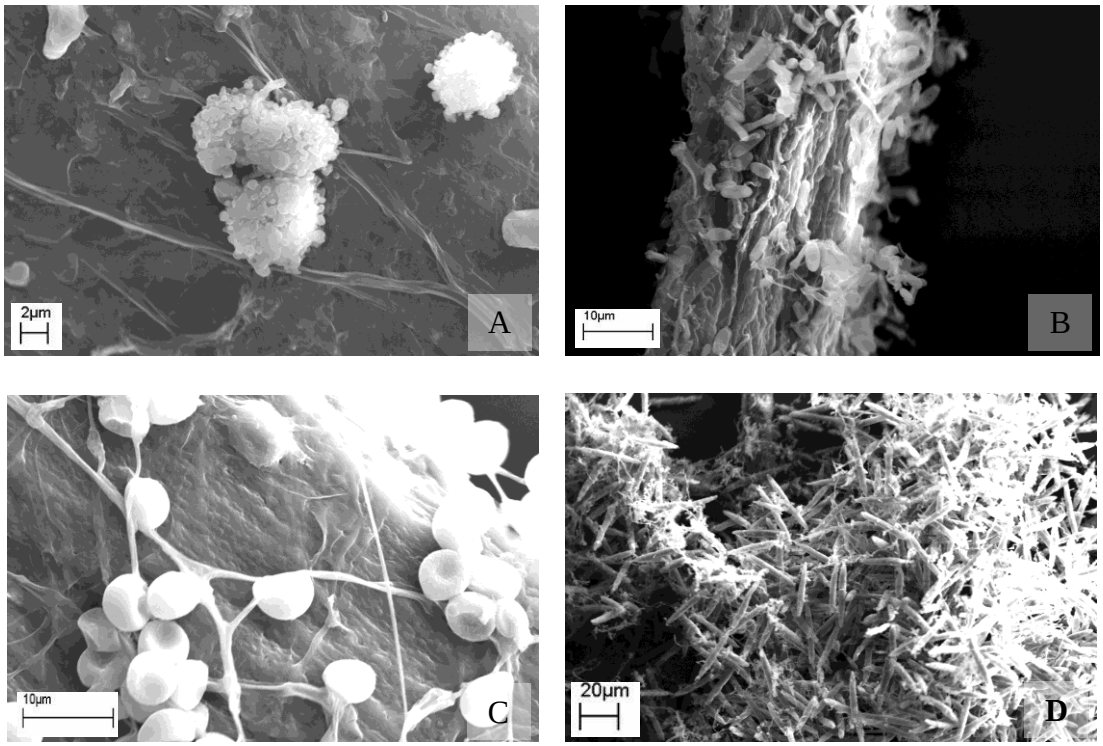

Figure 4.10: Morphology of fungal spores encountered during analysis of decomposing hair.

4.3.10 Morphology of fungal colonisations observed during SEM analysis of decomposing hair

The growth of fungi on decomposing hair resulted in several formations as illustrated in figure 4.11.

Figure 4.11A illustrated long hyphal structures growing along the length of the hair shaft. At the distal ends of the hyphae are possible fruiting bodies. Figure 4.11B shows the hair shaft covered in a fungus of a powdery constancy, whereas figure 4.11C shows the fungi growing in larger clusters along the length of the hair. Figure 4.11D shows the hair obscured by a 'hair like' fungal cluster that has intertwined in a complex mass. Finally, figure 4.11E illustrates a thread like hyphae that adhered closely to the hair shaft.

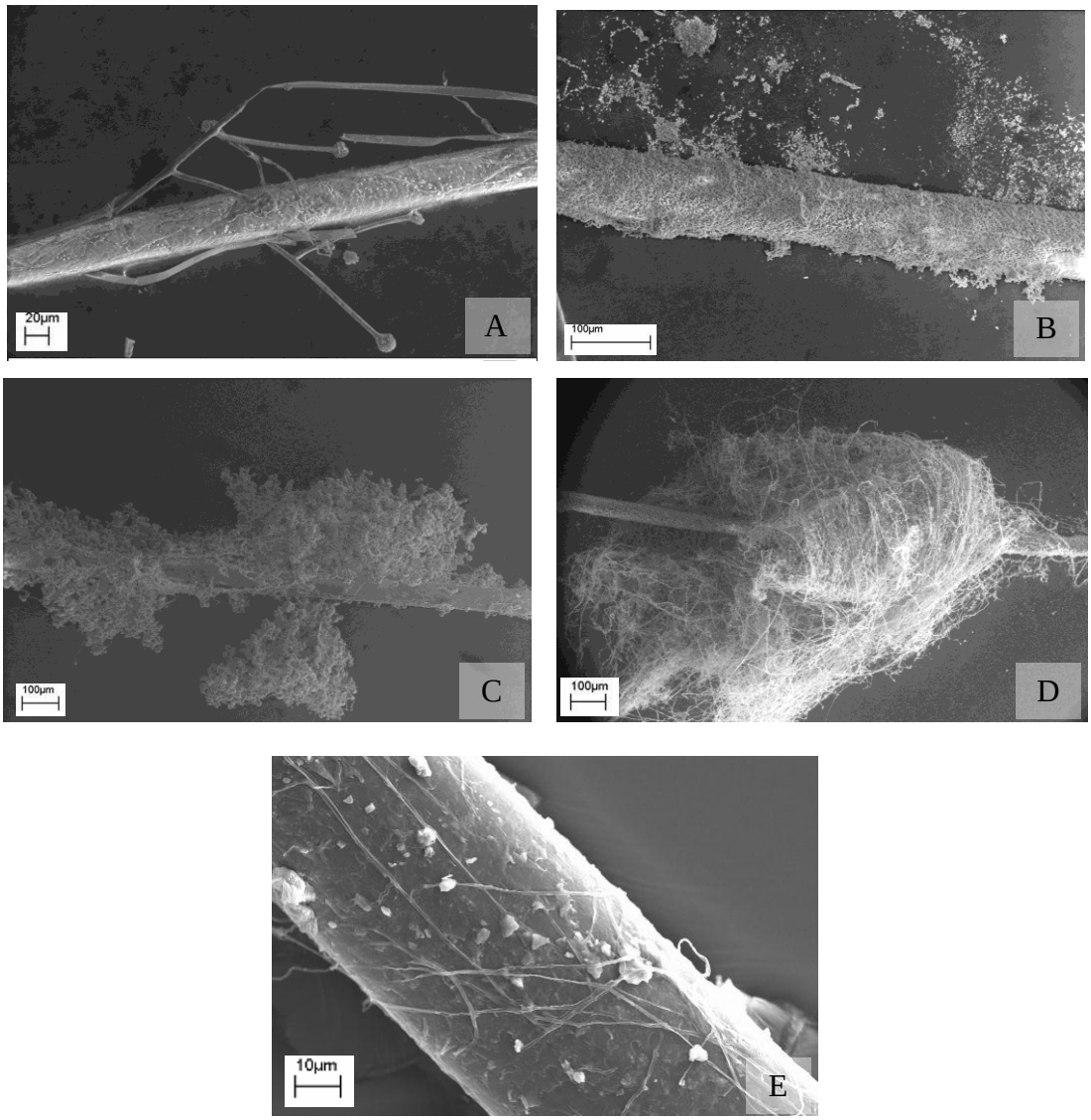

Figure 4.11: Different fungal morphologies encountered during scanning microscopy of decomposing hair.

4.3.11 DNA analysis of fungal species

Figure 4.12 shows the gel electrophoresis results for fungal DNA samples one to six. There were two lanes dedicated to each sample. DNA was present in lanes for samples one, two, three and four. However, DNA was only present in the first lane of sample five and the last lane for sample six. The DNA contained within each lane was visible as a streak progressing down the gel.

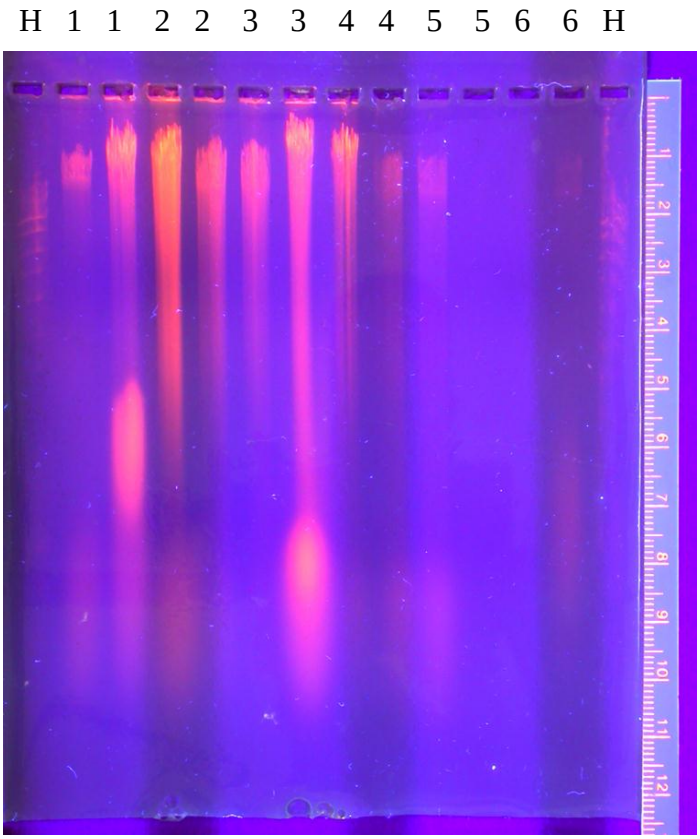

Figure 4.12: Gel electrophoresis of fungal DNA samples 1 to 6. Gel contains ethidium bromide. H: Hyperladder 1. There are two lanes for each sample.

Figure 4.13 shows the gel electrophoresis for fungal samples seven to 14. Each sample occupied two lanes. The hyperladder 1 had clearly separated into distinct bands. Most of the lanes contained a clear band of DNA. Samples seven to 11 had a clear band of DNA. Sample 12 exhibited bands although they were faint in comparison to the other samples in this gel. Sample 13 exhibited no band in the two wells. Sample 14 had clear bands of DNA.

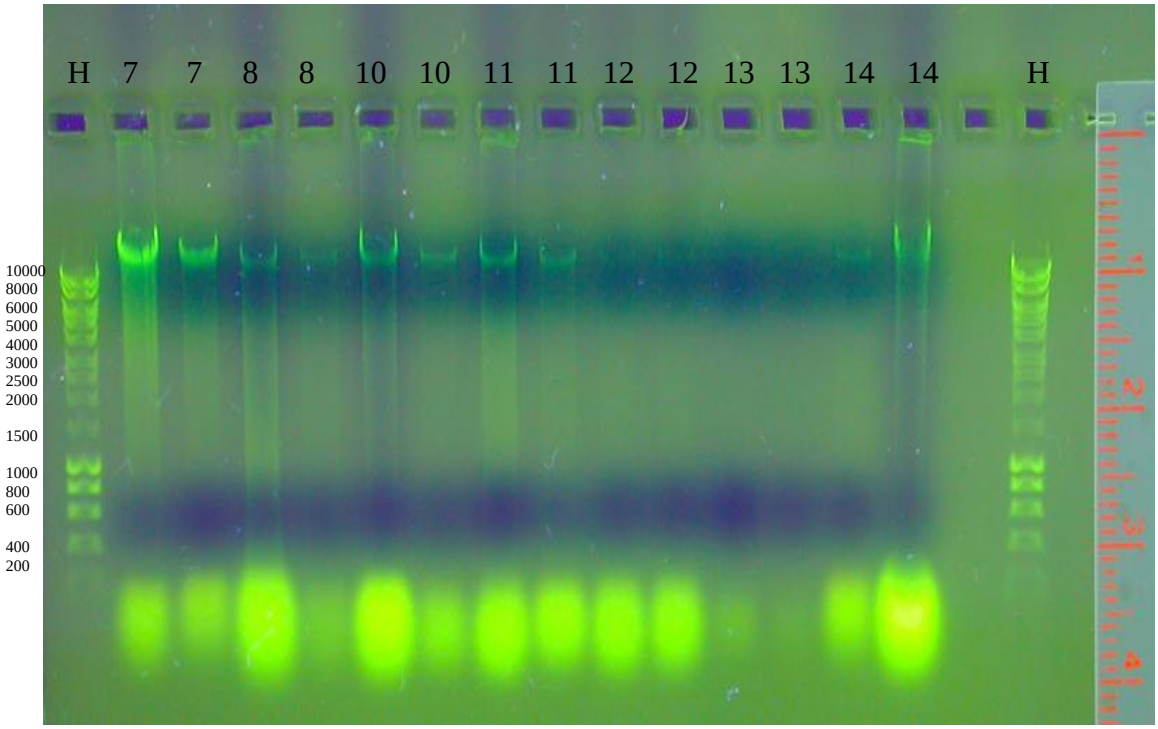

Figure 4.13: Gel electrophoresis of fungal DNA samples 7 to 14. The gel contains WebGreen. There are two lanes for each sample. H: Hyperladder 1.

Figure 4.14 shows the results for the gel electrophoresis analysis of fungal samples 15 to 20. Each sample occupied two lanes. It can be seen that all of the lanes contained a band of DNA. However, sample 15 contained faint bands. Furthermore, the second lane of sample 18 was also faint in appearance.

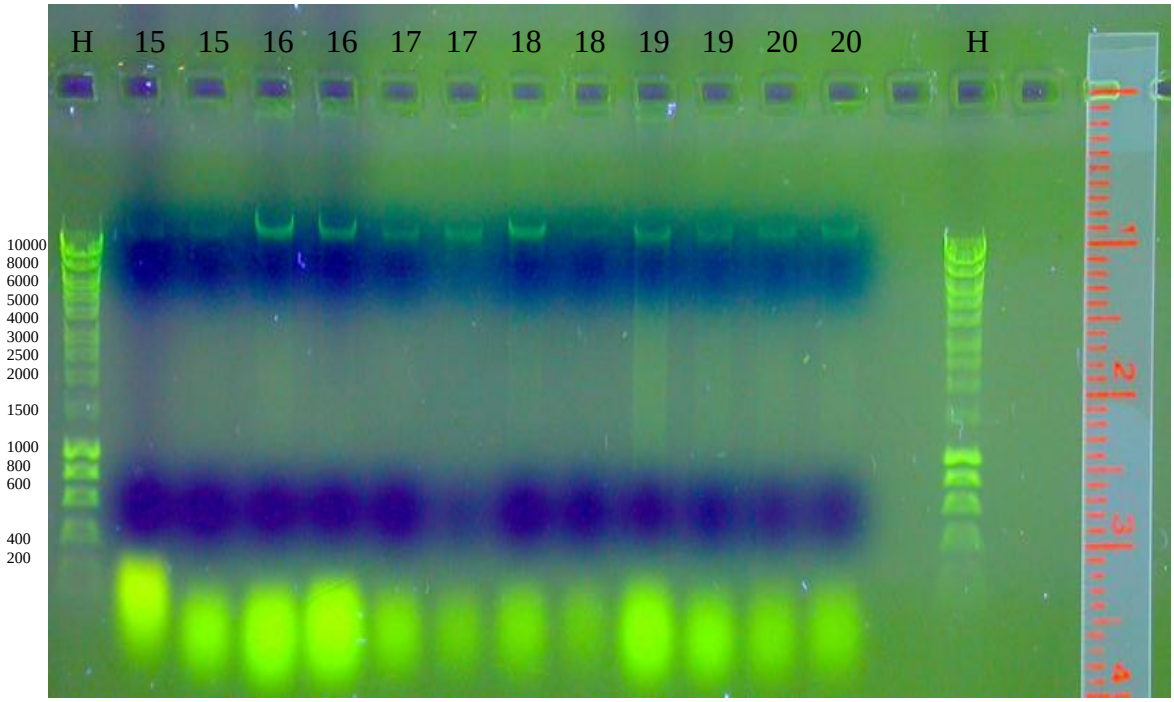

Figure 4.14: Gel electrophoresis of fungal DNA samples 15 to 20. There are two lanes per sample. Gel contains WebGreen.

The extracted DNA was subjected to amplification by PCR. Figure 4.15 shows the results for gel electrophoresis of the PCR products. Only sample one, three, five, six, seven, 10, 11 and 12 contained viable PCR product.

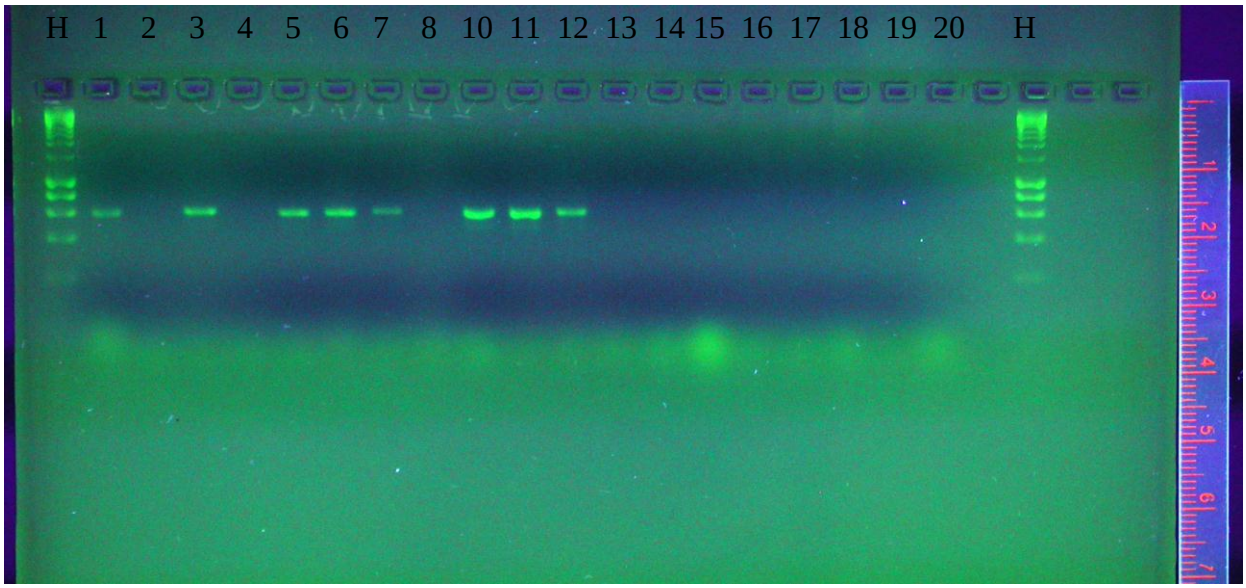

Figure 4.15: Gel electrophoresis of PCR products. Eight lanes contain viable PCR product. H: Hyperladder 1. Gel contains WebGreen.

Fungal DNA sequences were obtained from the DNA sequencing and Services at the University of Dundee. The fungal DNA sequences are detailed in Appendix 3.

By utilising BLAST, the eight fungal species were presumptively identified as *Aspergillus fumigatus* (PCR gel lanes: one, three, six, 10, 11 and 12) and a *Penicillium* species (PCR gel lanes: five and seven), see table 4.3. By comparison to other species in BLAST the *Penicillium* species could be *Penicillium chrysogenum*.

Table 4.3: The presumptive identifications of the fungal species collected.

PCR gel lane (figure 4.15)	DNA sequencing number (appendix 3)	Presumptive identification
1	1 and 2	*Aspergillus fumigatus*
3	3 and 4	*Aspergillus fumigatus*
5	5 and 6	*Penicillium* sp.
6	7 and 8	*Aspergillus fumigatus*
7	9 and 10	*Penicillium* sp.
10	11 and 12	*Aspergillus fumigatus*
11	13 and 14	*Aspergillus fumigatus*
12	15 and 16	*Aspergillus fumigatus*

4.4 Discussion

When interred in a shallow grave hair was subject to degradational changes. However, the occurrences of such changes were dependent on the characteristics of the depositional environment, namely, if the hair was with or without association to decomposing meat.

Degradational change included colonisation by fungi (e.g. figures 4.2, 4.3, 4.4, 4.5, 4.6 and 4.7) loss of cuticle scales (figures 4.4F, 4.6B, 4.7B, 4.9A), small and large erosions on the surface of the hair (figure 4.4 F and 4.9B), tunnelling formations within the hair (figure 4.8) and breaks to hair shaft (figure 4.6C and 4.9C). In one situation, when in a terracotta pot without exposure to meat (figure 4.6C), hair was reduced to a series of separated bundles and lost all of its characteristic features.

Macroscopic examination of the hair yielded limited and unspecific results, no damage to the hair shafts could be observed. Only on occasion, when large mycelial masses were present, could fungi be seen. Consequently the results were omitted from this work.

The utilisation of lactophenol cotton blue acted as a good preliminary indicator for the presence of fungi on the sample (figure 4.2 and 4.3). Furthermore, coupled with light microscopy, lactophenol cotton blue allowed for the visualisation of the internal aspects of the hair.

Scanning electron microscopy provided some interesting results, mainly focusing on the morphology of fungal structures and the resultant damage to the hair. It proved more useful than analysis with light microscopy due to its ability to analyse the hair surface. It must be noted however, that when fungal colonisation was extensive the surface of the hair was concealed. Different fungal morphologies were documented with the use of the SEM, these ranged from fungi with a powdery appearance to fungi which appeared as long hair-like masses (figure 4.11). In addition, various types of fungal spores were documented (figure 4.10). Interestingly, such visual information was absent in the forensic literature. During the SEM process, a vacuum is created within the SEM sample chamber and as a consequence fungal structures, especially spores, appear to have collapsed resulting in an altered appearance.

It was noted that the presence of decomposing meat had a great influence on the degradation process. Hair buried without exposure to meat degraded faster than when buried with meat (compare figures 4.6C and 4.7C), correlating with what is stated within the literature regarding decomposition limiting the effect of keratinolytic fungi (e.g. Wilson, 2008). This

phenomenon requires further investigation and understanding, especially considering that hair is usually found in association with decomposing bodies in shallow graves.

The tunnelling observed during the experiments conducted on water agar (figure 4.8) illustrated that while decomposing hair may seem architecturally sound on the exterior, the internal aspect may show the exact opposite as mentioned by Wilson *et al.*, (2001b). Furthermore, this experiment suggested that fungi are capable of tunnelling transversely through the hair shaft. While this has been documented in the literature, the samples on which the tunnels were documented were interred in soil. Consequently, there was always the possibility that the tunnelling was caused by or influenced by some other agent (e.g. soil). However, with no other known causative agents present in the water agar experiment, more evidence has been provided to suggest that fungi can form tunnels into the hair shaft. It was shown that tunnelling, in a non-competitive environment, is a phenomenon that occurs as time progress. However, only one fungus produced tunnels in all samples analysed between three weeks and 24 weeks.

DNA analysis of fungal samples presumptively identified *Aspergillus fumigatus* and a *Penicillium* species (most probably *Penicillium chrysogenum*) as fungi that colonised hair in a burial environment. *A. fumigatus* was encountered on all of the buried samples analysed, whereas the *Penicillium* was only encountered on the sample buried for 19 weeks in association with meat. Due to time constraints only one sequencing run was available. Further runs are required to highlight any sequences misreads, so at present the species level identifications are only putative. This research provided evidence that the possible *Penicillium* (*chrysogenum*) was the causative fungus for the tunnelling observed in figure 4.8. Both *Aspergillus* and *Penicillium* are universal fungi (Pitt, 1994). *Aspergillus fumigatus* is commonly found in soil environments (Soomro *et al.*, 2007) and has been reported to be keratinolytic and keratinophilic (Santos *et al.*, 1996 and Ulfig, 2003). Additionally, *P. chrysogenum* has been reported as a keratinophilic fungus on poultry bird feathers (Kaul and Sumbali, 2000).

The DNA analysis of colonising fungi allowed for a possible identification to the species level. This marks an advance in the application of fungi to the forensic arena as in previous publications identification was based on the morphology of fungi using light microscopy (Bunyard, 2004). However, due to identifying only two species, it was not possible to state if there was a successive fungal colonisation over time. Light microscopy for identification purposes offers limited value to the wider forensic community who are not experts in fungal

taxonomy, and if conducted by inexperienced analysts may result in misidentification (Bunyard, 2004).

The phenomenon of hair degradation within a soil environment is, based on these results, of very little value in the determination of a postmortem interval. Presently there are too many unknown variables greatly influencing the degradation process, variables including, the presence of decomposing matter along with moisture, pH and oxygen content. General thought would suggest that the more extensive the degradation the longer the burial interment. However, some conducted experiments showed extensive degradation and breakdown at 15 weeks (in a terracotta pot with exposure to meat, figure 4.6C) while others illustrated that the hair, other than some minor fungal growth, was morphologically and structurally sound at 24 weeks post burial (buried at Compton campus with meat, 4.5F).

Experimentation focusing on the effects of health and beauty treatments on hair needs to be conducted. Treatments such as hair straightening or blow drying may weaken the hair due to mechanical and thermal damage and as a consequence accelerate the degradation process. The effects of styling products such as hairsprays and gels which coat the hair in a substance will also need to be tested as these may offer protective qualities.

Head hair may act as a trap for pollen (Bryant *et al.,* 1990, Bryant *et al.,* 1998) and other particulates including fungal spores. This may result in initial colonisation if hair washing is not performed. Furthermore, damage by antemortem pathological fungi (Okuda *et al.,* 1988, Gupta, 2004), may also influence the way hair degrades after death. In addition, normal weathering processes weaken hair (see figure 4.16) and may provide direct internal access for fungi.

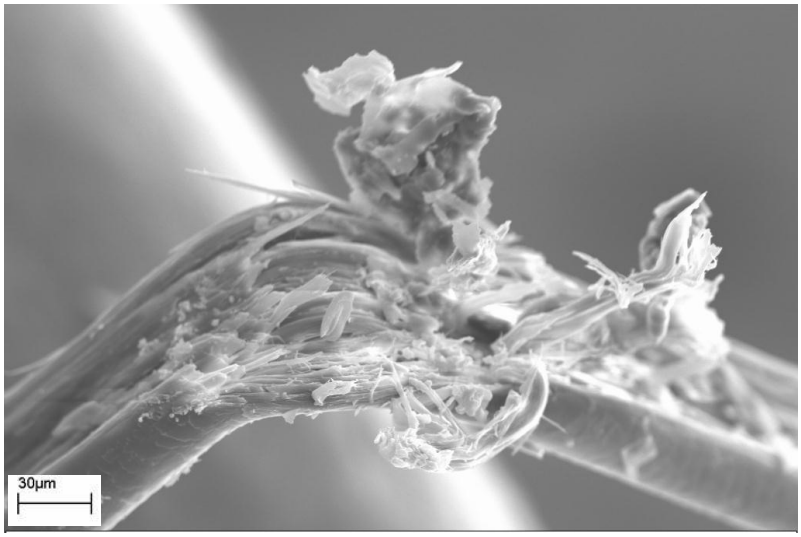

Figure 4.16: Weathering damage on human hair.

As already stated, the degradation of hair in a burial environment is of little use in determining the postmortem interval. However, the analysis of hair degradation could have other important forensic applications. DNA analysis, in the form of species specification, of fungi present on hair samples may help determine if remains have been buried, and may, after more in-depth research, help in determining the characteristics of the burial environment thus pinpointing possible locations. This could have important implications for the investigation of mass graves where remains may be transferred from primary to secondary or tertiary grave sites (Skinner *et al.,* 2003, Williams and Crews, 2003). However, for this application comparison between fungi from the remains and the burial site would be required. Although of little use in the context of determining a postmortem interval, any data pertaining to the decomposition and degradation of human remains or parts of should be welcomed by the forensic community.

Chapter 5: Bone

‘Taceant colloquia, effugiat risus. Hic locus est ubi mors gaudet succurrere vitae’.

‘Let conversations cease, let laughter flee. This is the place where death delights to help the living’ Latin proverb

5.1 Decomposition of Bone (Diagenesis)

The adult human skeleton is made up of 206 named bones (Krogman and Işcan, 1986, p.495, Glanze, 1990, Tortora and Derrickson, 2009, p.199, White and Folkens, 2000, p.29, Byers, 200, p.29) which in a living 70kg individual will weigh approximately 10kgs (Snyder *et al.*, 1976).

During life bone has many purposes, such as, protection of organs, support of soft tissues, allowing for movement and locomotion, act as a store for essential elements, and to act as a sink for waste or redundant elements (Armelagos *et al.*, 1989).

Bone is regarded as one of the strongest materials in biology (White and Folkens, 2000) and has three main components, a protein element (collagen), a mineral element (bioapatite, including hydroxyapatite), and ground substances (mucopolysaccharides and glycoproteins). The strength of bone holds great importance due to the constant forces that act upon it during life. For example, the forces applied when a person runs are equal to five times the weight of that person (White and Folkens, 2000). However, whilst being strong, bone also has a degree of elasticity (Antoine *et al.*, 1992).

The collagen situated in bone makes up 90% of the bones organic content (White and Folkens, 2000) and acts as a supporting structure to which the hydroxyapatite (HAP) is added and provides strength (Kumar and Clark, 1990). Hydroxyapatite is a crystal with a plate like morphology (Turner-Walker, 2008) that is located within the collagen matrix with their C axis aligned parallel to the long axis of the collagen fibres (Turner-Walker, 2008). Consequently the collagen and hydroxyapatite are joined by powerful molecular bonds which help give strength and ensure preservation of bone under ideal conditions (Dent *et al.*, 2004). This characteristic makes bone a composite material (White, 2000).

Most mammals house two types of bone, trabecular (cancellous or spongy bone) and cortical (or compact bone) (Antoine *et al.*, 1992). This is represented in figure 5.1.

Figure 5.1: A partially sectioned human femur illustrating bone anatomy (Tortora and Grabowski, 2000).

Cancellous, trabecular or spongy bone has, as the name suggests, a porous morphology consisting of a network or trabeculae (Antoine *et al.*, 1992). This type of bone is mainly situated at the proximal and distal aspects of a bone and found below compact or cortical bone (Antoine *et al.*, 1992).

Bone being a mix of collagen and bioapatite, (Child, 1995, Collins *et al.*, 2002) decomposes, although the process of bone decomposition can take thousands of years under favourable conditions (Dent *et al.*, 2004). Diagenesis, in its most basic form, is any natural modifications that occur to bone postmortem, and can alter the type of information contained within the bone itself (namely isotopic, molecular, biochemical and structural information (Hedges, 2002).

Bone becomes unstable when removed from the body (Truman *et al.*, 2004) because it moves from a relatively closed environment to an open one (Turner-Walker, 2008) and can be broken down over time via numerous processes, including physical breaking, decalcification, and dissolution due to acidic soil and water (Dent *et al.*, 2004).

When buried, bone changes. These changes can involve uptake of materials from the soil, the exchange of ions, the breakdown and leaching of collagen, microbiological attack, alteration and leaching of the mineral matrix and infilling with mineral deposits (Hedges, 2002). Changes to osteological material from a burial environment can be analysed using light microscopy in the first instance (Herrman, 1993), although further techniques such as SEM

and TEM (transmission electron microscopy) could be used. Grupe and Dreses-Werringloer, (1993) suggest a functional and practical approach to analysis of bone diagenesis. As bone is normally sectioned for determination of age at death and pathology, for ease and cost effectiveness the same samples can be used for decomposition research (Grupe and Dreses-Werringloer, 1993).

The presence or absence of water in the immediate environment surrounding bone will influence its degradation (Turner-Walker, 2008). Water is the medium for chemical reactions and supports microbial activity (Turner-Walker, 2008). The presence of water around bone may, depending on pH, causes dissolution of its mineral components. Furthermore, the presence of water containing ions (such as calcium (Ca^{2+}), phosphate (PO_4^{2-}) and carbonate (CO_3^{2-})) may result in alterations to the structure of HAP (Hedges and Millard, 1995). Nicholson (1996) explains that in free draining soils, degradation of bone is accelerated as soluble materials are lost. Furthermore, the opposite would occur in waterlogged soil, where the persistent presence of water creates a buffered local environment as solutes are held by the surrounding ground water, thus slowing down decomposition. This phenomenon is further explained by Turner-Walker (2008) who states that the size of bone pores will influence the quantity and locality of water infiltration in bone. However, once saturated, Ca^{2+} and PO_4^{2-} will dissolve. In waterlogged environments this will result in localised dissolution and overall bone preservation will be good. However, in an environment where bone enters a wet and dry cycle, the pore water containing Ca^{2+} and PO_4^{2-} will be drawn out by a hydraulic potential. After wetting again, water will enter the bone pores and once again become saturated with Ca^{2+} and PO_4^{2-} (Turner-Walker, 2008). When the bone dries this water will be lost along with its ions and the bone enters a wet verse dry cycle 'positive feedback loop' which will result in a decrease in preservation rates (Turner-Walker, 2008).

The effect of sediment pore water on the mineralogical and compositional changes in bone on soil surface is discussed by Trueman *et al.,* (2004) who found that water flow is governed by evaporation in a process which they term as 'analogous to the wicking of a candle'.

The pH of groundwater will further enhance the effect of water on bone as calcium phosphates (including HAP) become soluble as the pH falls (Lindsay, 1979, Turner-Walker, 2008). Furthermore, the pH of water will also influence what ions are present. In acidic burials, bone becomes discoloured due to transitional metal ions and humic acids becoming soluble and invading bone tissue. Conversely, in alkali, soils bone becomes cream in colour due to the locking up of metal ions as insoluble carbonates and oxyhydroxides (Turner-

Walker, 2008). The pH, and elemental composition of the soil, may result in the formation of Vivianite, Brushite and Calcite within bone (Child, 1995). Details of Brushite formation are presented by Piepenbrink (1989). In addition to degrading HAP, extremes of pH can result in the hydrolysis of collagen (Collins *et al.*, 2002).

A high presence of Ca^{2+} within soil (depending on pH) may result in the destruction of bone as explained by Piepenbrink (1989) and Child (1995), Calcium carbonate ($CaCO_3$) formed in soil with high calcium content occupies greater space than crystals of HAP and may result in internal stress within the bone subsequently manifesting as cracking. Similar processes occur with the formation of Brushite in acidic soils (Piepenbrink, 1989).

The activity of microbes will also result in the degradation of bone. In 1864, Wedl found, by accident, that a collection of teeth in his possession had been colonised by a possible fungus. He noted microscopic tunnelling within the teeth and further experimented by immersing teeth in a media inoculated with the original microbe. Upon analysis he noted penetration into the teeth after 13-17 days of immersion. He conducted similar experiments using animal bone and documented the same tunnelling structures.

Fungal growth on bone has been reported by Piepenbrink (1986, 1989) who was able to isolate four species of fungi from exhumed bone. In one of the most recent papers to date, Hawksworth and Wiltshire (2010) were able to obtain 13 fungal isolates from the human skeletal remains in a forensic context. The identification of these fungal species has only been published in a police report which offers no accessible contribution to the discipline. Grupe and Dreses-Werringloer (1993), found that numerous inoculated fungi and bacteria were able to survive on sterilised bone. The fungi included: *Botrytis* sp., *Cladosporium cladosporiodes*, *Cladosporium herbarum*, *Cladosporium* sp, *Crysosporium pannorum*, *Cyrsosporium* sp., *Penicillium brevi-compactum*, *Penicillium herbarum*, *Phialophora hoffmannii* and *Scytalidium* sp. The bacterial species utilised included: *Actinomadura madurae*, *Alkaligenes pichaudii*, *Arthrobacter globiformis*, *Bacillus subtilis*, *Pseudomonas fluorescens*, and, *Streptomyces griseus*. The authors found that colonisation was dense after four months but the integrity of the bone was uncompromised with no observable tunnelling (Grupe and Dreses-Werringloer, 1993).

A study worthy of note was conducted by Hackett (1981), who defined the term microscopical focal destruction (MFD). He found, on bone buried for one year, evidence of tunnelling. Hackett's reasoning was that microorganisms present in the soil progressed into the bone via endosteal and periosteal routes and surmised that the microorganisms fed on

collagen via systematic demineralisation. Hackett developed a classificatory system for his microbially induced histological changes: Wedl tunnels, linear longitudinal tunnels, budded tunnels and lamellate tunnels. In a recent publication Jans *et al.,* (2004) state that linear longitudinal, budded and lamellate tunnels observed by Hackett were caused by bacteria.

Microbial attack on bone has been discussed in detail by Hedges (2002), who states that, at present, too little is known of microbial attack for a model to be developed. The author states that degradation by microbes is highly variable and can result in differences in preservation within and between archaeological sites.

While it has been stated that bacteria and fungi are present within the soil and may infiltrate into the bone, Bell *et al.,* (1996) propose that postmortem, bacteria from the gut can make their way into Haversian canals via nutrient arterials and venous vasculature. This idea was also suggested by Nicholson (1998), who states that bone destruction is not a straightforward process starting at the outside and progressing interiorly. The architecture of bone will influence how bacteria are able to colonise. Turner-Walker (2008) explains that bacteria are unable to cross cement lines but some species can travel longitudinally along bone and seem to follow the orientation of collagen fibres. The penetrating microorganisms will survive feeding on the surrounding unmineralised tissue and excrete metabolites which will vary according to the local environment (Child, 1995). Under anaerobic conditions, the decomposition of proteins will result in a decrease in pH due to the production of amino acids and fatty acids (Child, 1995). The presence of these in bone will result in the demineralisation of HAP and expose collagen to collagenases (Child, 1995). It is crucial that the HAP is lost as bacteria are unable to degrade mineralised collagen (Turner-Walker, 2008). It is also important that HAP is lost leading to an increase in porosity, allowing large collagenases to enter further in to the bone (Nielson-Marsh *et al.*, 2000a and b). The loss of the mineralised and organic components leaves the bone porous and fragile and, if such processes continue, the bone will ultimately disintegrate and be lost.

Grupe and Piepenbrink (1989) were able to prove that microorganisms could carry metals from the soil into bone specimens in a laboratory setting. Furthermore, Grupe *et al.,* (1989) provided some, albeit limited, evidence suggesting that microorganisms may cause isotopic fractionation, especially occurring in nitrogen isotopes within a bone sample.

Nicholson (1998), observed the decomposition of a mix of animal bone in a compost heap. The author reports bone surface modifications consisting of discolouration (namely reddish yellow to reddish brown) the colouration was analysed by comparison with Munsell colour

chart. The author also reports yellow and white fungal species which, unfortunately, were not identified. Fungal hyphae were observed on the bone focusing around the epiphysial margins. Pits with smooth edges were also observed along with tunnels which caused the cortical bone to separate from the main bone body. Further analysis by histology confirmed the presence of fungal hyphae, which were also observed at the endosteal surface (Nicholson, 1998). In addition to soil conditions and microbiological activity, bone is also modified by the activity of plant roots, scavengers and intentional and accidental anthropogenic modifications (Cox and Bell, 1999).

Of particular note in relation to this research are the similar observations of microbial attack seen between human and animal bones as reported by Hedges (2002) and Ezzo (1994). This needs further investigation ideally with a comparison experiment conducted on both animal and human bone material of the same anatomical location and in similar environments, exposed to similar variables and excavated at the same time intervals.

5.2 Methods

5.2.1 Bone provenance

Individual pig bones were used to analyse taphonomic phenomena. In a burial environment it is likely the long bones will survive longer than the bones of other classifications if conditions are favourable. This is due to denser mineralisation in the bones thick, cortical structure (Ezzo, 1994).

The bones were collected from a butcher located on the outskirts of Andover, Hampshire. The bones were in the form of whole defleshed pig legs. These were stored in a freezer (-10°C) until ready for processing. The bones, namely the femur and tibia were cut using a hacksaw in to approximately 1 inch cross sections, and then frozen until interment commenced.

Interment of the bone sections was conducted on the 5th February 2008. Table 5.1 shows the specific data for burial excavations and durations. As with all of the samples for this research burial was conducted in shallow graves, at Compton Campus, to simulate a forensic situation.

Table 5.1: Excavation and burial duration data for porcine tibial and femoral cross sections.

Date of Excavation	Burial duration (Months)
5th March 2008	1
4th April 2008	2
5th May 2008	3
5th June 2008	4
7th July 2008	5
5th August 2008	6
6TH October 2008	8
5th December 2008	10
5th February 2009	12
7th April 2009	14
6th July 2009	17
5th October 2009	20
4th December 2009	22
4th February 2010	24

5.2.2 Macroscopic analysis of bone cross sections

Macroscopic analysis to observe decompositional phenomena was conducted. Changes in colour and texture along with any adhering features, namely fungi, plant or insect materials were recorded and documented using photography (Finepix Z, Fujifilm). An additional light source was utilised to highlight any changes in more detail.

5.2.3 Sample dissection

Four longitudinal sections, approximately 4 cm x 1 cm (to obtain a representative overview of the entire sample), of the buried bone samples were dissected for SEM analysis using a circular power saw with a diamond coated blade (saw: 300 series, Dremel. blade: 545 diamond wheel, Dremel). To ensure adequate health and safety, the bone was cushioned in paper towels and placed into a metal clamp ready for dissection (figure 5.2A). Dissection was made by two vertical lines that follow the longitudinal direction of the bone (Figure 5.2B). These sections of bone were then halved (to ensure they fit into the SEM), stored in a plastic vial and frozen until ready for analysis.

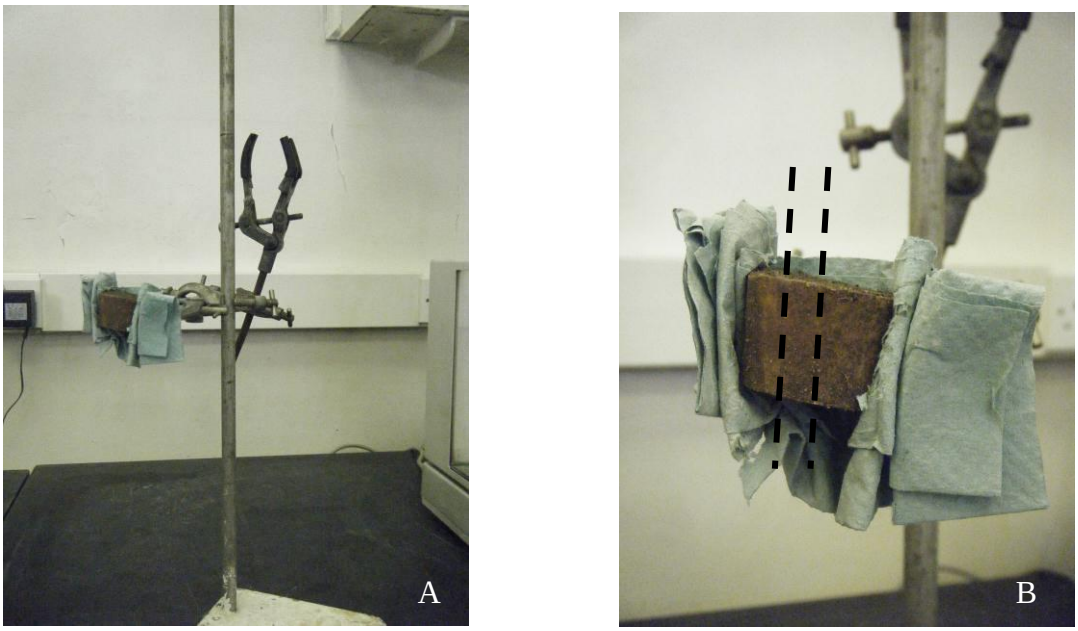

Figure 5.2: Equipment set up for bone dissection. A illustrates the overall set up, B shows that the bone is cushioned in paper towel to prevent crushing. The lines illustrate the directionality of the cut marks.

5.2.4 SEM analysis of bone samples

Bone was attached to adhesive carbon padded stubs and placed directly into the SEM (EVO 50, Zeiss). On occasion gold coating was required to obtain clearer images, however this was not a routine procedure. Magnification was varied depending on the findings during analysis.

5.3 Bone results

5.3.1 Macroscopic analysis of bone

Two images detailing the longitudinal and transverse aspects are presented for each of the cross sections for the femur and tibia samples. Table 5.1 presents a summary of all the macroscopic finding.

Figure 5.3 shows the morphology of a section of control femur. Soft tissue is still in association with the bone surface. The bone is ivory in colour. Bone marrow is present in the medullary cavity which was red in colouration and had a jelly like consistency. No fungal or plant structures were observed and erosions were absent.

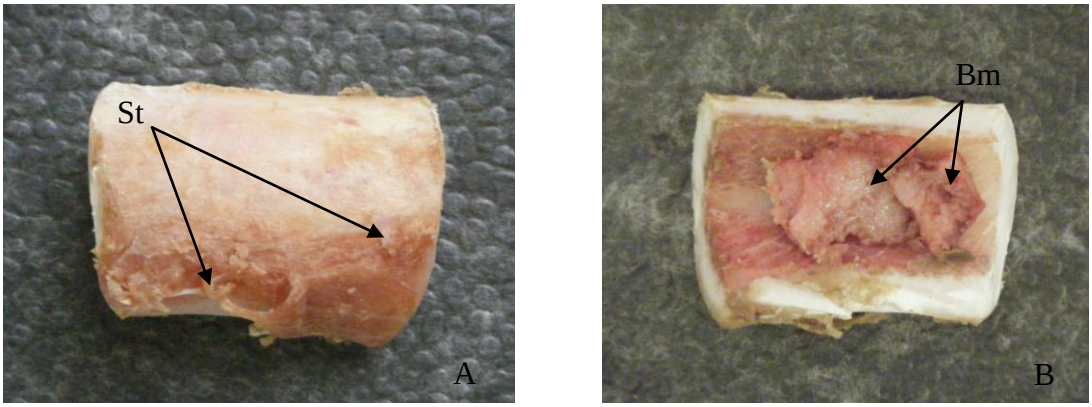

Figure 5.3: Control femur. A: external aspect, B: internal aspect. St: soft tissue, Bm: bone marrow

Figure 5.4 illustrates the femur at one month post burial. There was no observable degradation to the sample surface. Furthermore, bone marrow was still present within the medullary cavity which was brown in colouration. The bone surface was ivory in colour. Soil was present, adhering to the outer compact bone. Plant material, in the form of roots, was found on this sample, but their abundance was limited.

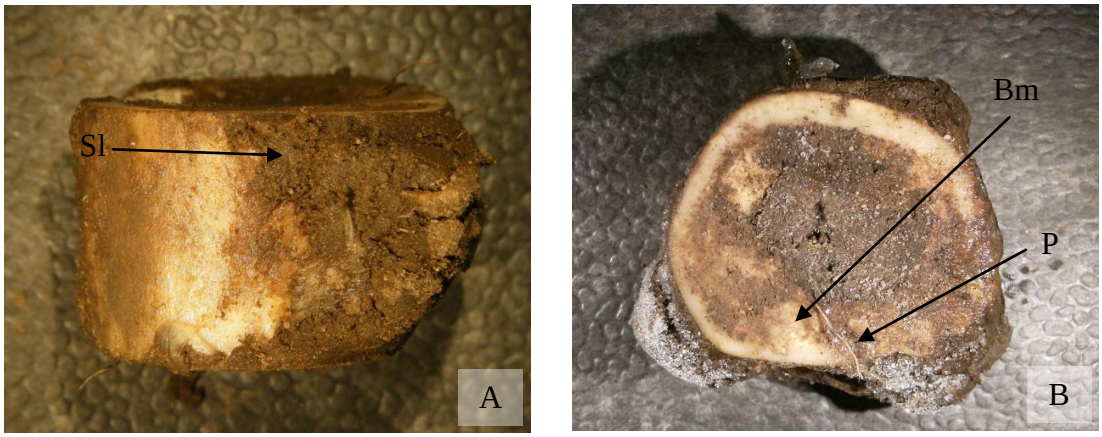

Figure 5.4: Femur one month post burial. A: longitudinal, B: transverse views. Bm: bone marrow, P: plant material, Sl: soil.

Figure 5.5, shows femoral bone at two months post burial. Entomological activity was noted on this sample, which was most likely feeding on the residual soft tissues. The bone surface, after cleaning, was noticed to be undamaged and ivory in colour. Fungal activity, in the form of white mycelia, was documented on the bone marrow. The bone marrow was brown in colour.

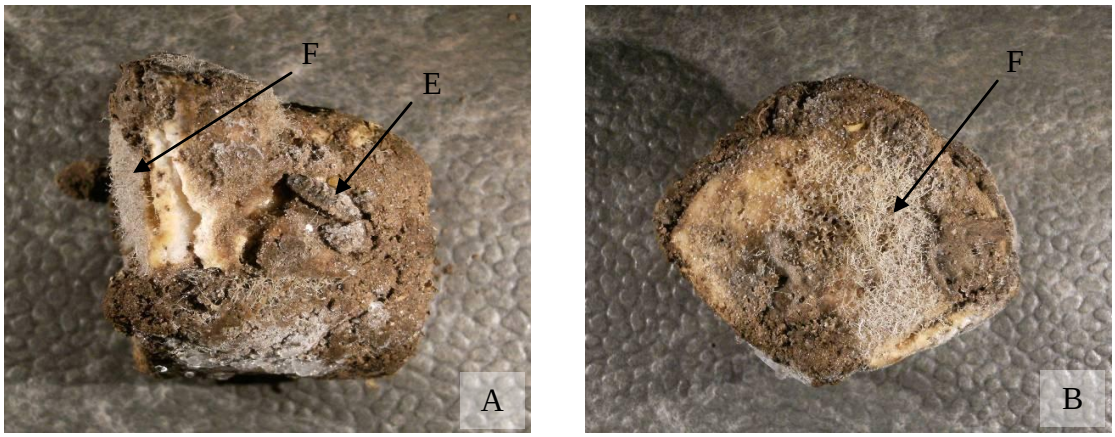

Figure 5.5: Femur two months post burial. A: longitudinal, B: transverse views. E: entomology, F: fungi.

Bone at three months post burial is illustrated in figure 5.6. The surface of the bone exhibited no damage and it was ivory in colour. There was bone marrow still present in the medullary cavity with fungal structures still in association. As with the earlier samples, the bone marrow was brown in colour. Plant matter was beginning to grow on the bone surface.

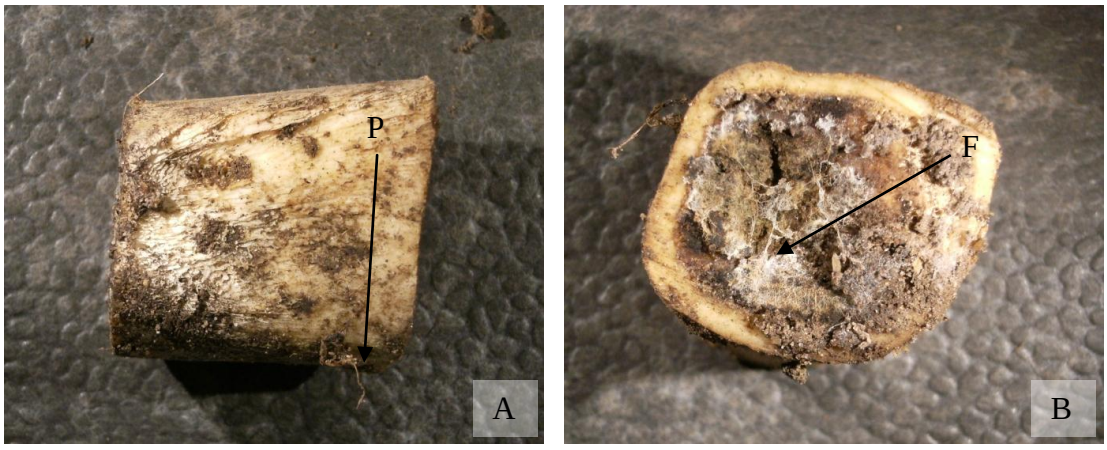

Figure 5.6: Femur three months post burial. A: longitudinal, B: transverse views. F: fungi, P: plant material.

After four month post burial the bone surface was beginning to show some erosions as seen in figure 5.7.A. The erosions manifested as a rough and scored texture to the surface of the compact bone. These erosions could be due to the extensive plant growth evident on the sample. Most of this growth was situated around the transverse cut in close association with the remaining bone marrow which was white in colour. The bone surface was ivory in colour. No fungal structures were found on this sample.

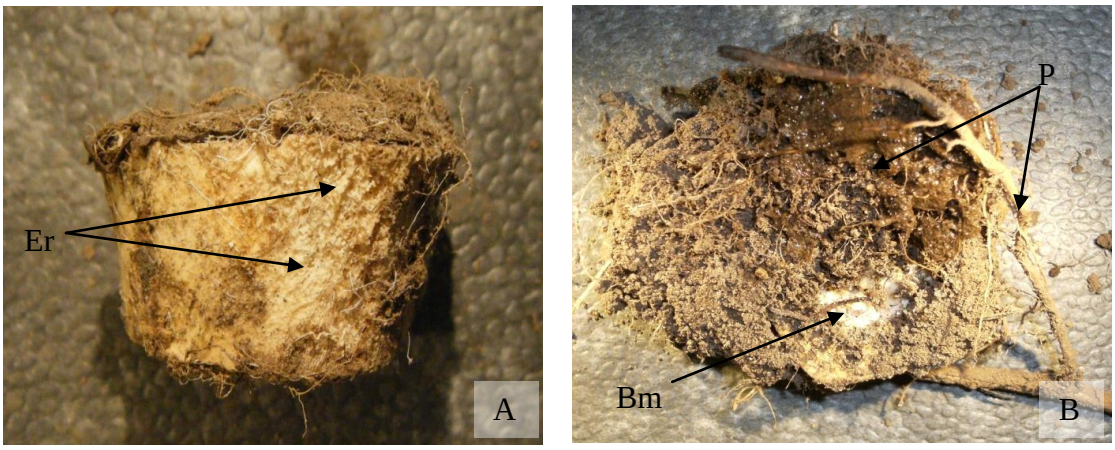

Figure 5.7: Femur four months post burial. A: longitudinal, B: transverse views. Er: erosions, Bm: bone marrow, P: plant material.

The Five month sample (figure 5.8) exhibited some plant growth although this was greatly reduced compared to the four month sample (figure 5.7). The plant growth was observed as thin roots on the surface of the compact bone. The bone marrow was absent with the resultant void becoming filled with soil. The bones surface exhibited some erosion manifested as a rough, uneven surface. These erosions were localised and not widespread on the sample. The surface of the bone was ivory in colour. Furthermore, fungi were absent from this sample.

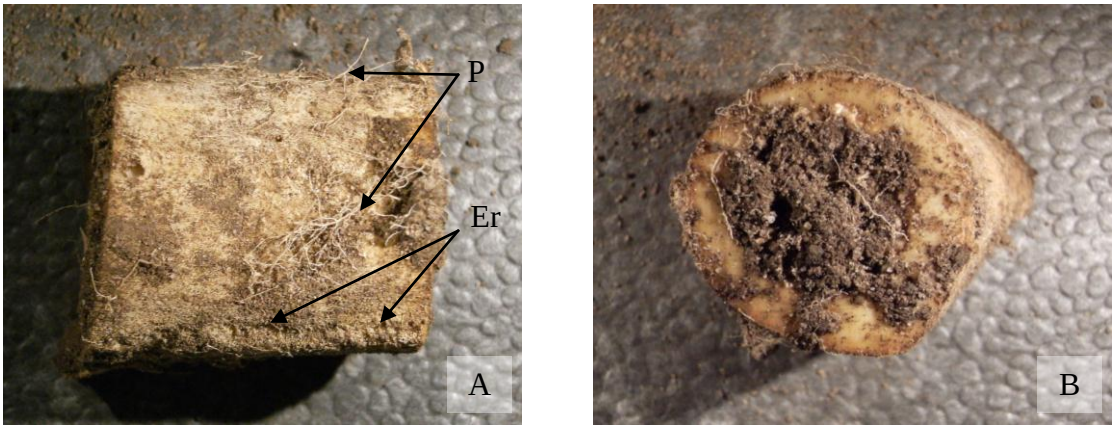

Figure 5.8: Femur five months post burial. A: longitudinal, B: transverse views. Er: erosions, P: plant material.

Figure 5.9 illustrates a femoral sample at six months post burial. The bone surface was smooth and appeared uneroded compared to the four and five month sample (figures 5.7 and 5.8). Once again the surface of the bone was ivory in colour. The quantity of bone marrow was reduced within the medullary cavity and it had become white in appearance. Little soil was also present in the medullary cavity. There were some plant structures present around the transverse section. These were thin and root like in morphology. Fungal structures were absent.

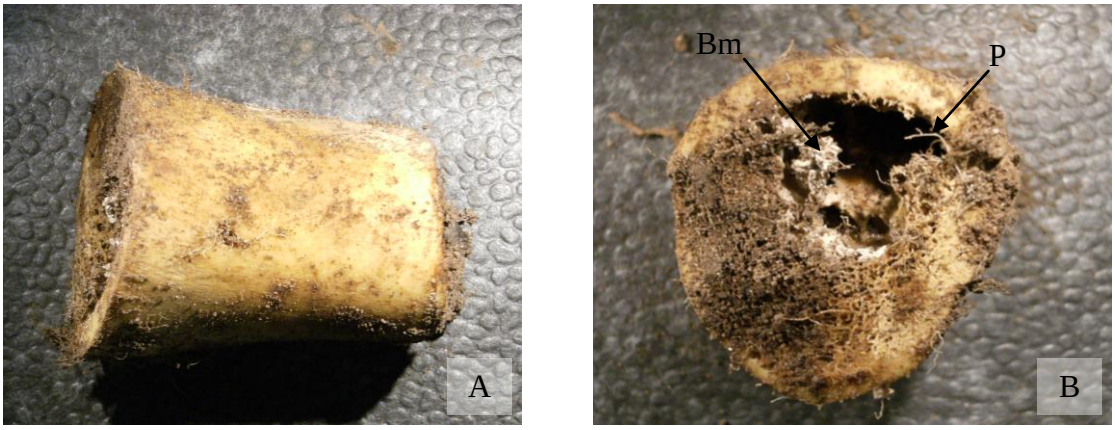

Figure 5.9: Femur six months post burial. A: longitudinal, B: transverse views. Bm: bone marrow, P: plant material.

Figure 5.10 details a bone sample at eight months post burial. Plant material was still in association and the bone surface was smooth in appearance and uneroded. Additionally, the surface was ivory in colour. Soil had infiltrated the medullary cavity. Futhermore, a white

fungus was visable on the bone surface, this was different in morphology from the fungi seen on the samples in figures 5.5 and 5.6 (two and three month samples respectivly).

Figure 5.10: Femur eight months post burial. A: longitudinal, B: transverse views. F: fungi, P: plant material.

Contrary to the previous samples, figure 5.11 shows that after 10 months post burial the bone had darkened probably due to interaction with the burial environment. Cortical surface erosions were absent from the surface of the bone and soil was present in the medullary cavity. A white fungus was observed on the surface of the bone (figure 5.11A). A small amount of plant material was also present on the transverse section of the sample.

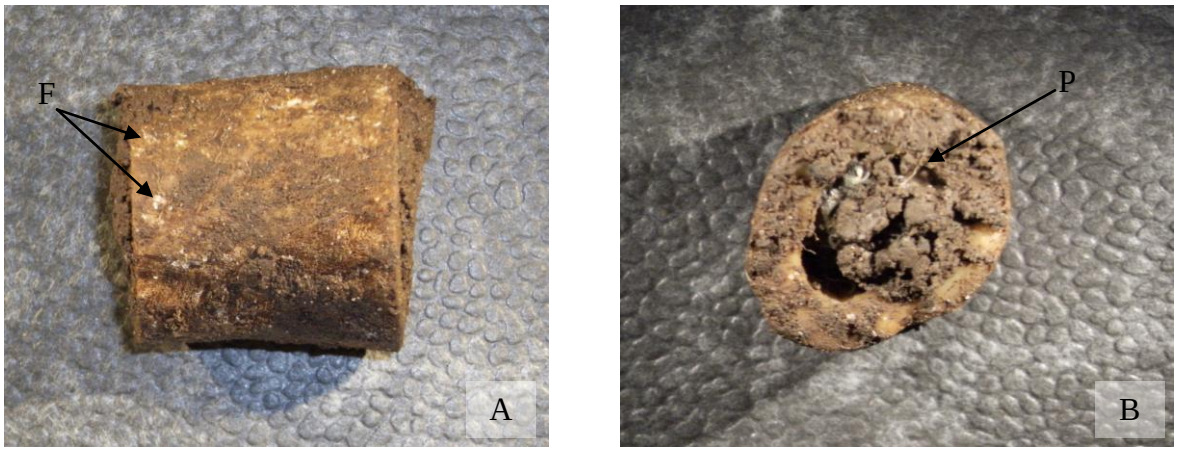

Figure 5.11: Femur 10 months post burial. A: longitudinal, B: transverse views. F: fungi, P: plant material.

At 12 months post burial the sample appears to have some erosive damage to the surface of the cortical bone, this appeared as a grainy texture and was widespread on the sample (figure 5.12). The surface of the bone was ivory in colour. Soil was present inside the medullary cavity. The white structure found on the soil in the cavity was a web like structure resultant of

insect activity. The area of missing bone was resultant of sample harvesting for SEM analysis. Some plant material was also noted.

Figure 5.12: Femur 12 months post burial. A: longitudinal, B: transverse views. Er: erosions, P: plant material.

At 14 months post burial (figure 5.13) the sample exhibited some modification to the cortical bone surface. This was manifested as erosions with a grainy texture which were widespread across the sample (figure 5.13A). The surface of the bone was light ivory in colour. Some plant material was present. The medullary cavity was vacant of bone marrow and soil when the sample was excavated. The area of missing bone was due to sample collection for SEM.

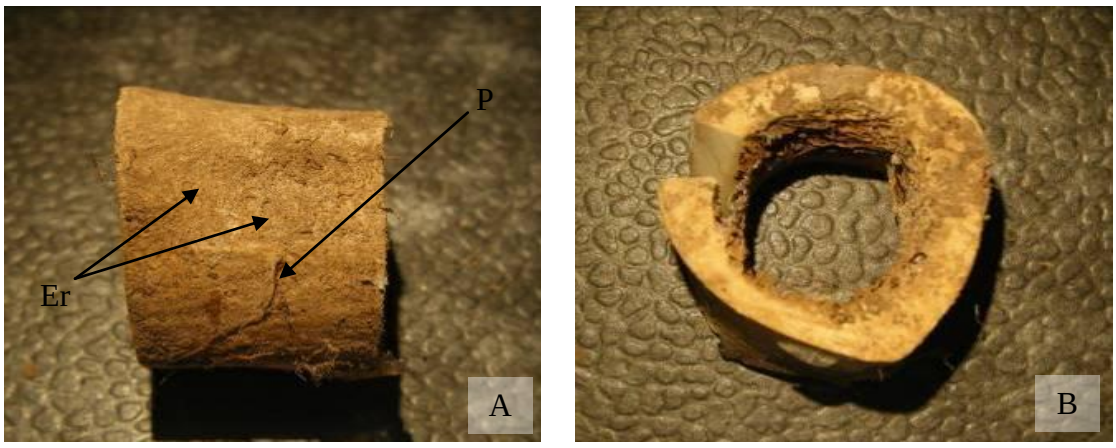

Figure 5.13: Femur 14 months post burial. A: longitudinal, B: transverse views. Er: erosions, P: plant material.

Figure 5.14 illustrates a femoral sample at 17 months post burial. Plant material in the form of roots was present on the bone surface. The cortical bone was ivory in colour but had areas beginning to darken. The medullary cavity was void of bone marrow and full of soil. The surface of the bone appeared smooth and uneroded. Fungi were absent from the surface of the sample.

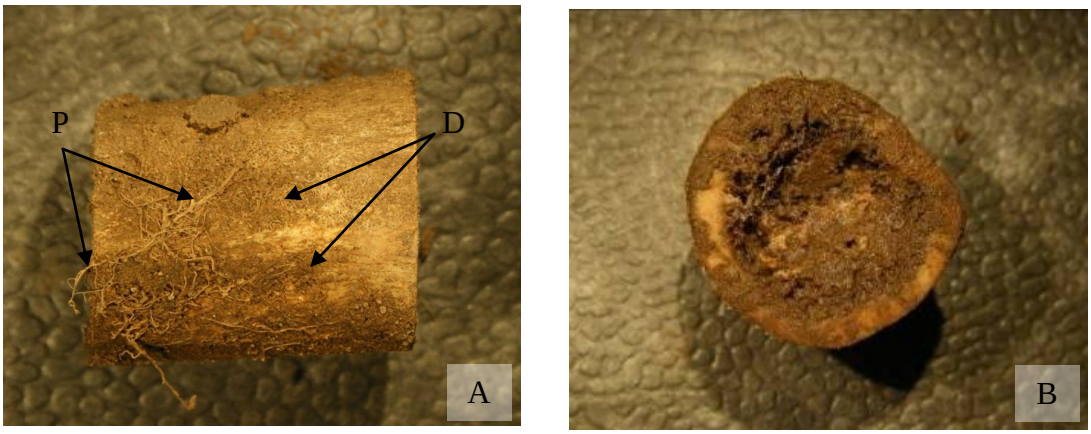

Figure 5.14: Femur 17 months post burial. A: longitudinal, B: transverse views. P: plant materials. D: darkened areas.

At 20 months post burial (figure 5.15) the surface of the bone was in good condition with no visible erosions. The surface of the bone was dark in colouration. As with previous samples (e.g. four months: figure 5.7, five months: figure 5.8, six months: figure 5.9, eight months: figure 5.10, 14 months: figure 5.13 and 17 months: 5.14), plant material was in association on the surface of the sample. Upon excavation it was noted the medullary cavity was void of bone marrow and soil. No fungal activity was observed.

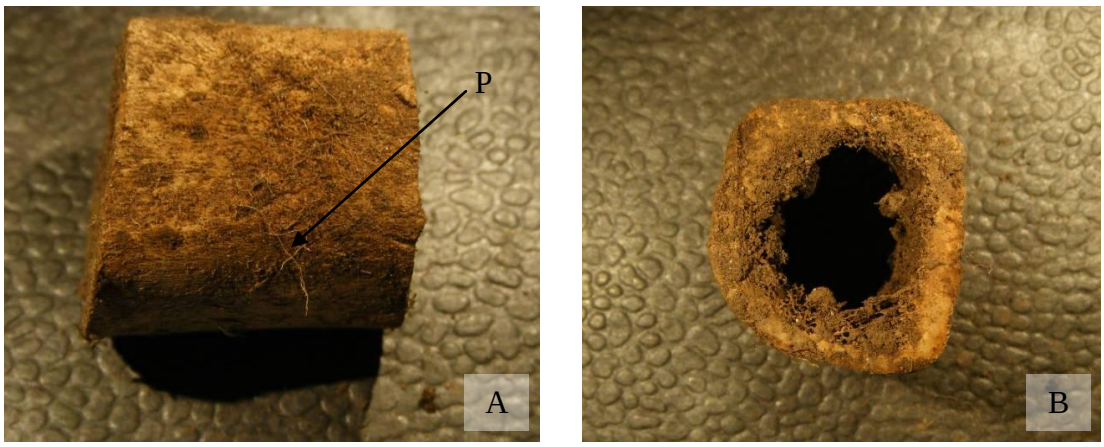

Figure 5.15: Femur 20 months post burial. A: longitudinal, B: transverse views. P: plant material.

After 22 months of burial the surface of the sample was covered in a white fungus (figure 5.16). Whereas this fungus had been documented in previous samples (eight months: figure 5.10 and ten months: figure 5.11), it's prevalence on the 22 month sample was greater. In addition, plant matter was documented and the medullary cavity was occupied with soil. The cortical bone was dark in colour but lacked any modification.

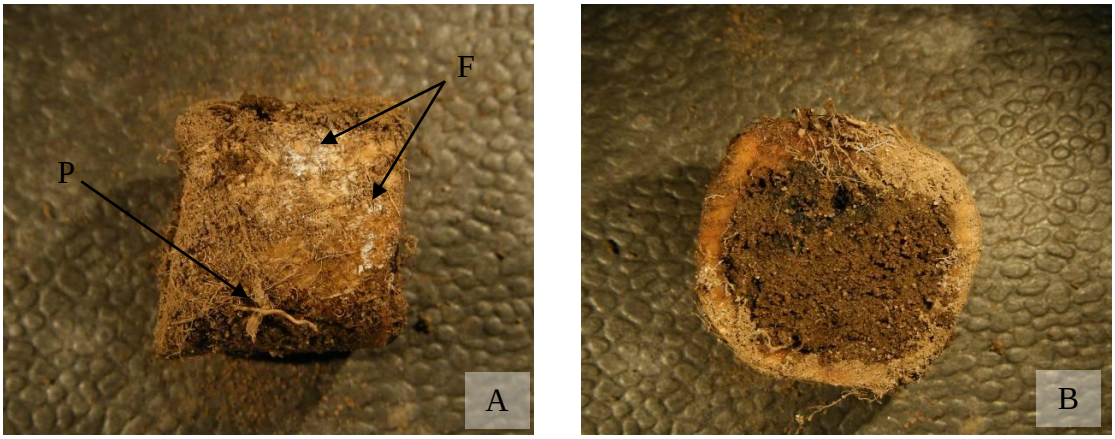

Figure 5.16: Femur 22 months post burial. A: longitudinal, B: transverse views. F: fungus, P: plant material.

The sample buried for 24 months exhibited little modification, in the form of erosions, to the bone surface (figure 5.17). However, some erosive damage was noted. This was in the form of a grainy and irregular texture. The cortical bone was dark in colour. Fungal structures were absent from the sample. Plant matter was present although this was limited. As with the previous samples, the medullary cavity was occupied with soil.

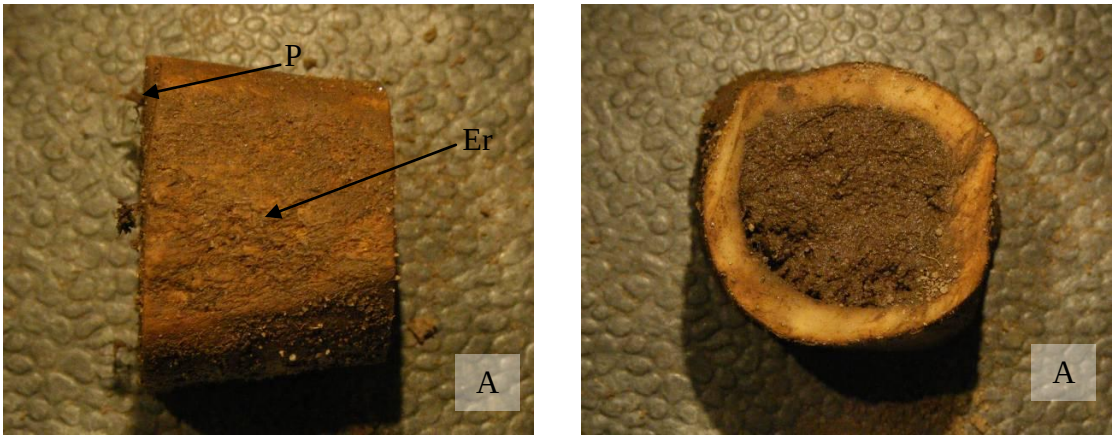

Figure 5.17: Femur 24 months post burial. A: longitudinal, B: transverse views.

Tibia

Figure 5.18 shows the control tibia sample. It can be seen that the surface was ivory in colour and still retained some soft tissue (figure 5.18A). Analysis of the internal aspect (figure 5.18B) of the sample revealed red bone marrow. The surface exhibited no plant or fungal growth and no erosions were documented.

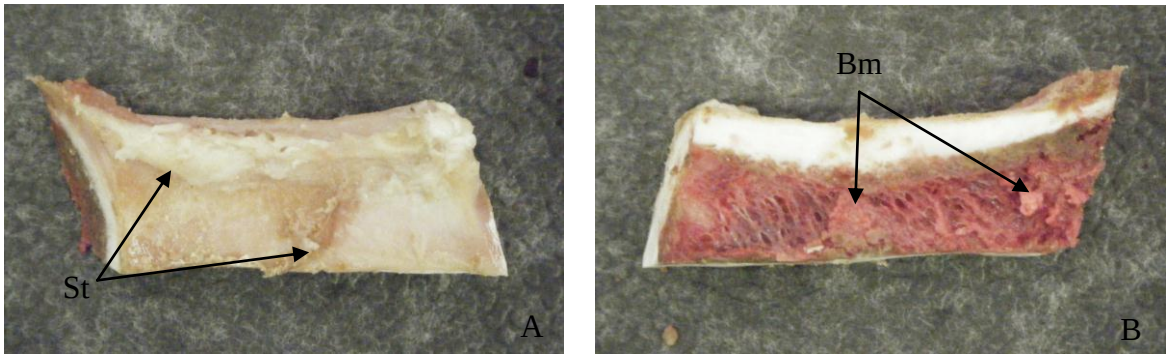

Figure 5.18: Control tibia sample. A: external aspect, B: internal aspect. St: soft tissue, Bm: bone marrow.

Figure 5.19 details a section of tibia one month post burial. The bone's surface was smooth with no evidence of pitting. It was ivory in colour but still retained soil from the burial. There was plant material in association in the form of small roots. The area of missing bone in figure 5.19B was due to sample harvesting for further analysis. Bone marrow was still present in the medullary cavity and it was white in appearance. Fungal structures were absent.

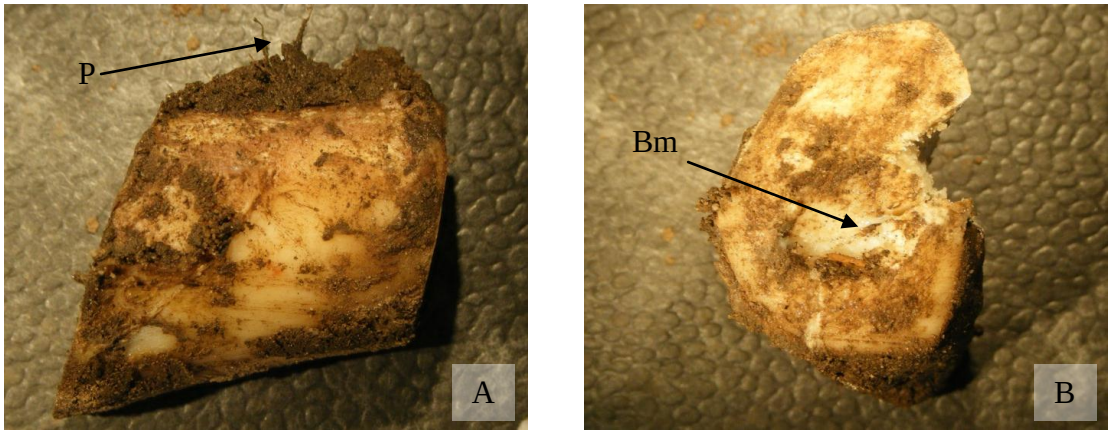

Figure 5.19: Tibia one month post burial. A: longitudinal, B: transverse views. P: plant material, Bm: bone marrow.

Figure 5.20 details a tibial section at two months post burial. It appears from figure 5.20B that the bone marrow was beginning to degrade. It was white in appearance but was becoming reduced in quantity within the cavity. The bone surface was smooth with no evidence of degradation. The surface was ivory in colour. Some plant material was present on the sample, again manifested as root structures. Fungi were absent from this sample.

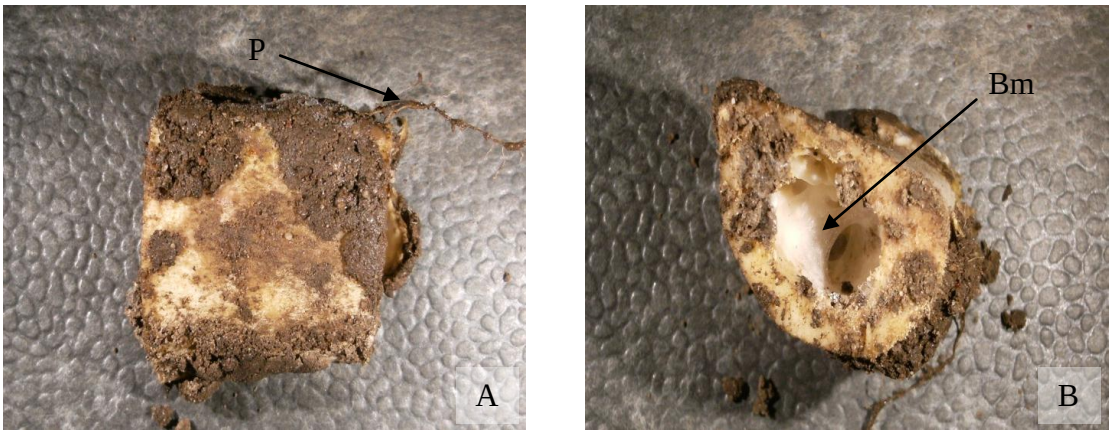

Figure 5.20: Tibia at two months post burial. A: longitudinal, B: transverse views. P: plant material, Bm: bone marrow.

At three months post burial the bone marrow within the medullary cavity had degraded and soil had begun to infiltrate into the available space (figure 5.21). The bone surface exhibited no degradation changes and was ivory in colour. Some plant material was present although this was minimal. No fungi were observed on this sample.

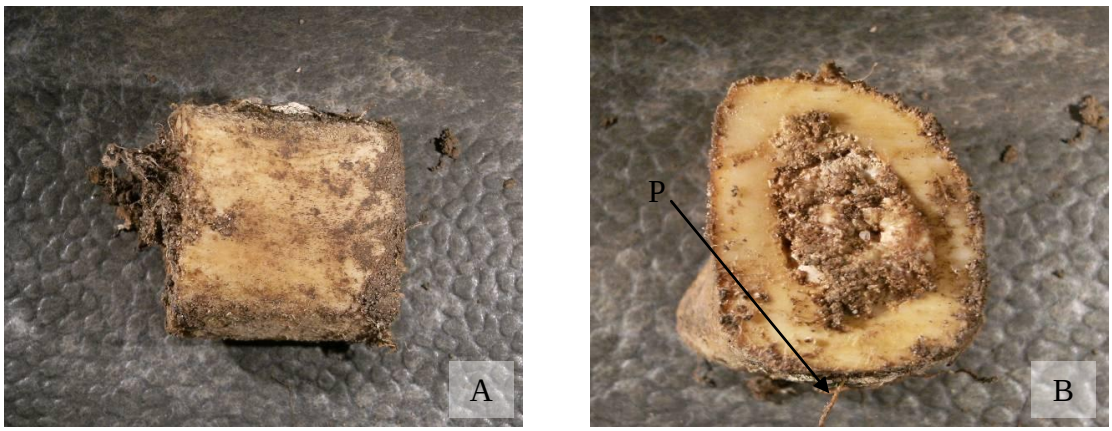

Figure 5.21: Tibia three months post burial. A: longitudinal, B: transverse views. P: plant material.

At four months post burial, (figure 5.22), the sample had some fungal growth similar in morphology to the fungus seen on the femoral samples in figures 5.5 and 5.6. Otherwise, the bone was presented in good condition with no sign of degradation or erosions. As with the previous tibial samples the surface was ivory in colour. The medullary cavity was devoid of bone marrow but did contain a small amount of soil. The sample was absent of any plant material.

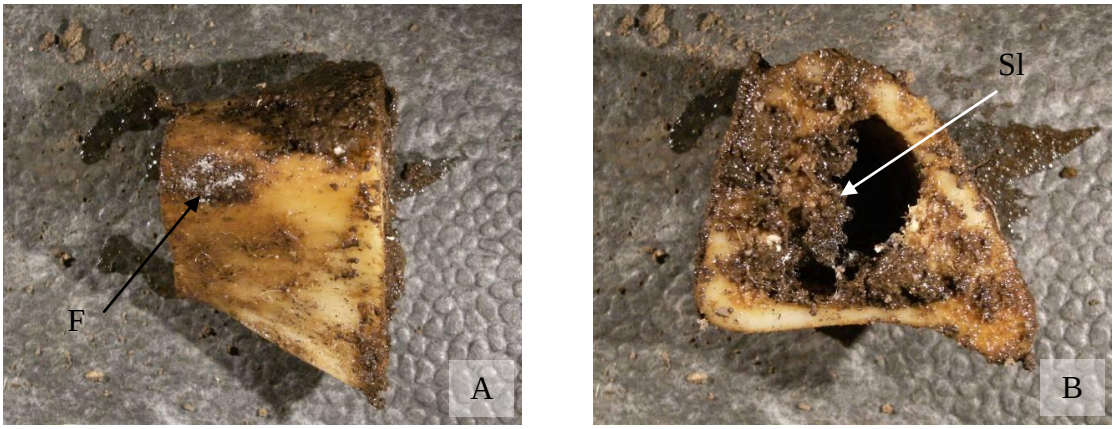

Figure 5.22: Tibia four months post burial. A: longitudinal, B: transverse views. F: fungi, Sl: soil.

At five months post burial there was some erosional changes to the bones surface (figure 5.23). These changes were observed as a grainy texture with some apparent scoring. The surface of the sample was ivory in colour. There were some plant rootlets present on the sample but fungal activity was absent. The medullary cavity was filled with soil.

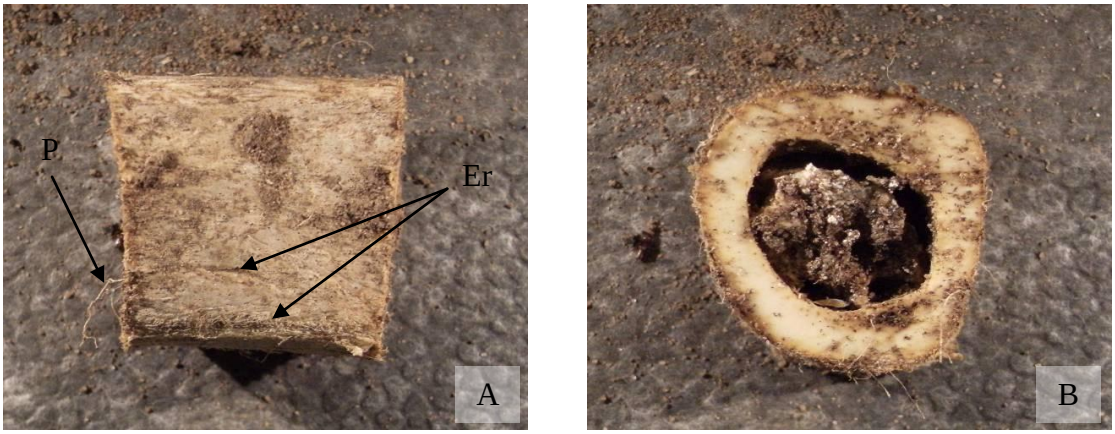

Figure 5.23: Tibia five months post burial. A: longitudinal, B: transverse views. P: plant material, Er: erosions.

The degradational changes observed on the six month sample (figure 5.24) were pronounced with the surface exhibiting erosions and scoring with a grainy texture. These erosive modifications were widespread. Plant growth was situated at the transverse section in the form of a dense network of roots. Overall, the sample was an ivory colour and the medullary cavity was occupied with soil.

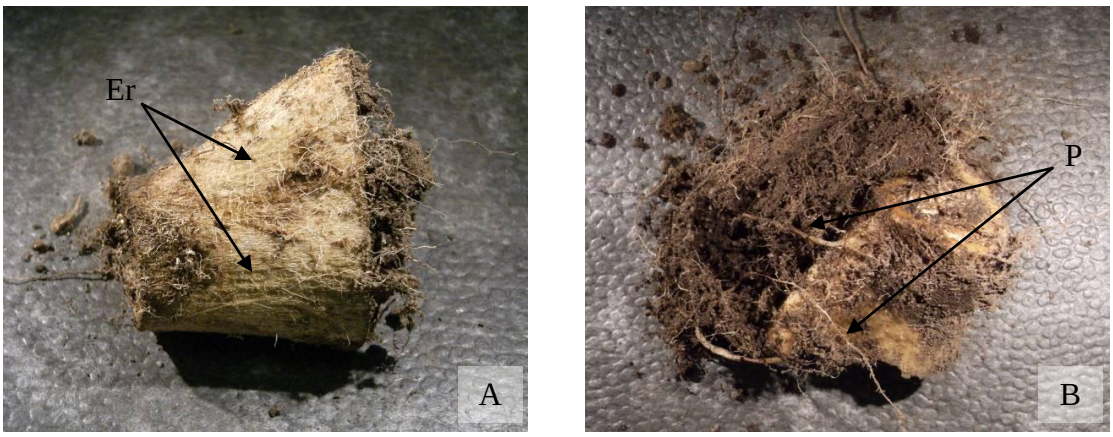

Figure 5.24: Tibia six months post burial. A: longitudinal, B: transverse views. Er: erosions, P: plant material.

Fungal growth was observed on the sample buried for eight months (figure 5.25). The quantity of the fungi was pronounced and it was widespread on the sample surface. The bone surface was an ivory colour with no sign of damage. There was little plant material in association with this sample. The medullary cavity was empty of bone marrow but was partly occluded with soil.

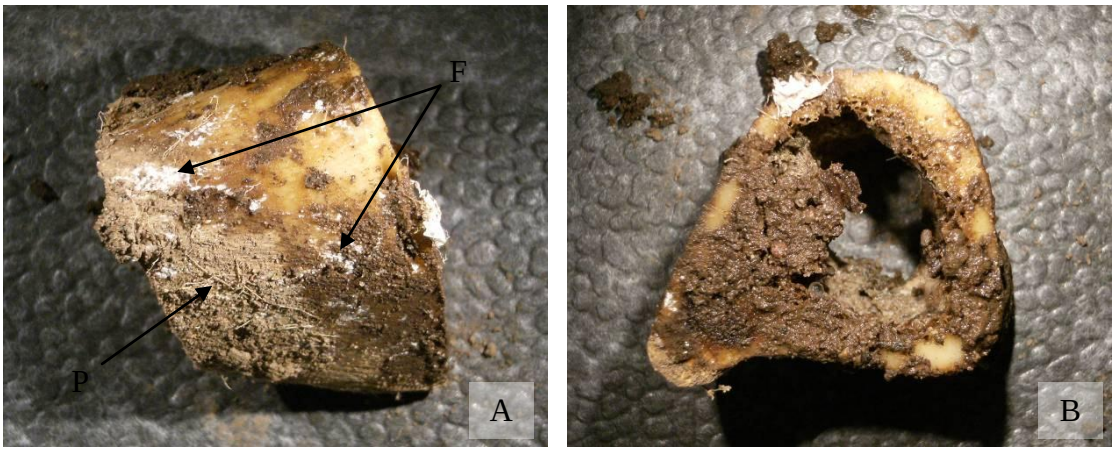

Figure 5.25: Tibia eight months post burial. A: longitudinal, B: transverse views. P: plant material, F: fungi.

Figure 5.26 details a section of tibia interred for 10 months. The surface of the sample was in good condition compared to the six months sample (figure 5.24). The surface was absent of erosive degradational changes and was smooth in appearance. It was also an ivory colour. There was a small amount of fungal activity observed on the bone surface but this was localised. The medullary cavity was occluded with soil. Only a small amount of plant material, in the form of roots, was observed with this sample.

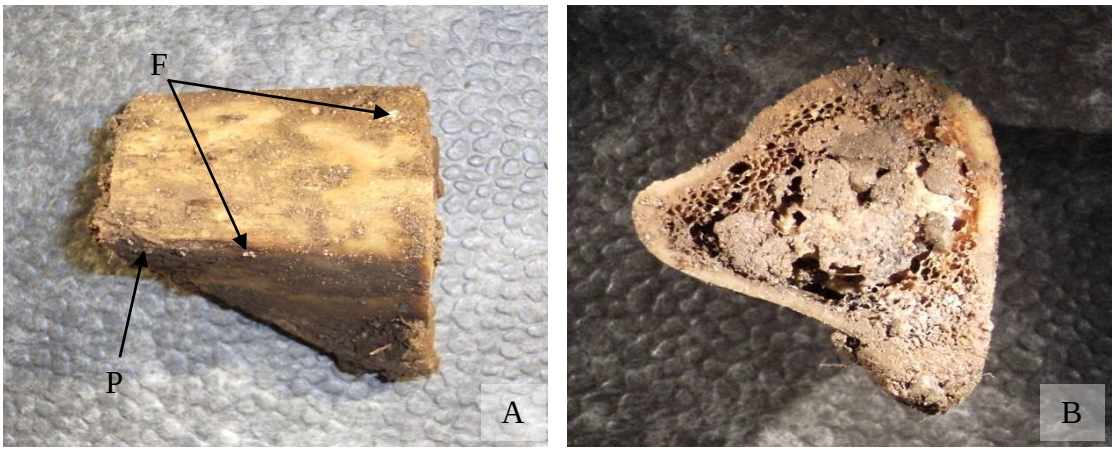

Figure 5.26: Tibia 10 months post burial. A: longitudinal, B: transverse views. F: fungi, P: Plant material.

At 12 months post burial, the tibial sample exhibited associative plant growth to the bone surface (figure 5.27). Otherwise, the bone appeared to be in good condition with a smooth and uneroded surface, with an ivory colour. The area of missing bone in figure 5.27B was due to sample harvesting for further analysis. The medullary cavity was empty of soil and bone marrow upon excavation. A small area of fungal activity was noted on the transverse section of the sample.

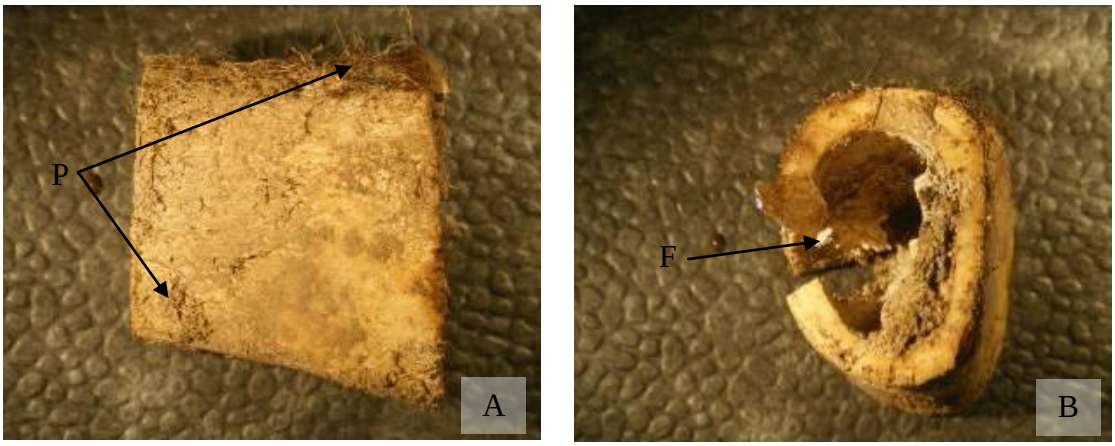

Figure 5.27: Tibia 12 months post burial. A: longitudinal, B: transverse views. P: plant material, F: fungi.

Figure 5.28 exhibits a tibial section at 14 months post burial. Other than some plant growth, the bone surface appears uneroded and lacking any scoring. The area of missing bone seen in figure 5.28B was due to sample harvesting for further analysis. The bone remained an ivory colour, comparable to the previous samples. No fungal activity was observed.

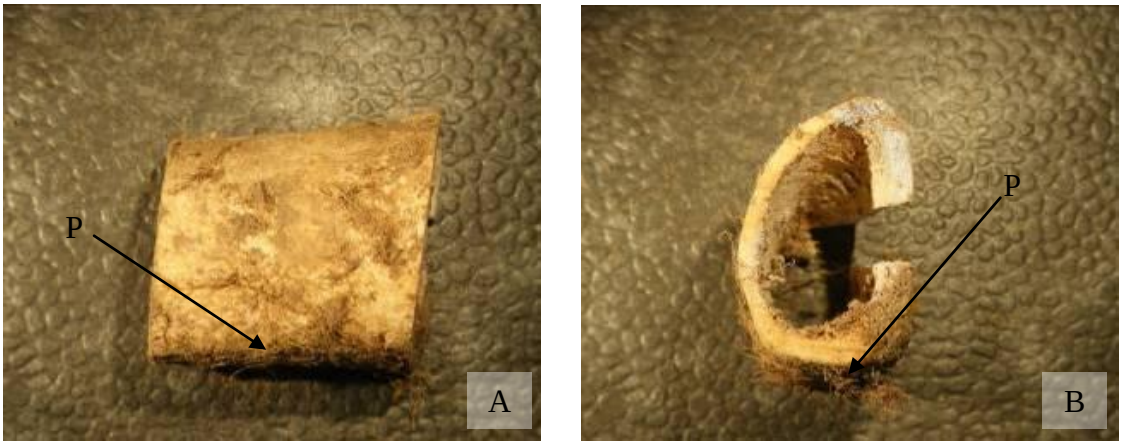

Figure 5.28: Tibia 14 months post burial. A: longitudinal, B: transverse views.

The bone surface was in good condition after 17 months of burial, lacking any erosions or scoring (figure 5.29). However, contrary to the previous samples, it was dark in appearance, likely due to interaction with the burial soil (Figure 5.29A). A white fungus, similar to the fungus previously seen (four months: figure 5.22, eight months: figure 5.25 and 10 months: figure 5.26 samples), was present. Little plant material was documented on this sample and the medullary cavity was occupied with soil.

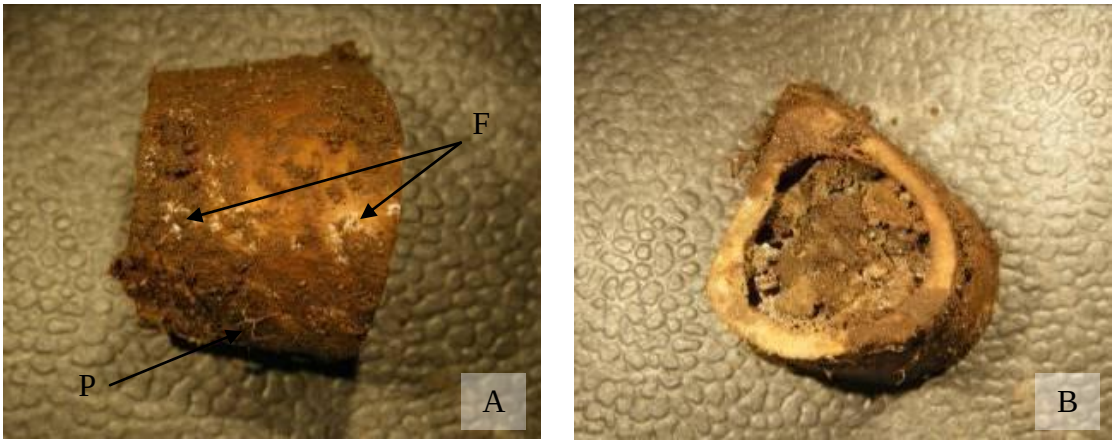

Figure 5.29: Tibia 17 months post burial. A: longitudinal, B: transverse views. F: fungi, P: plant material.

Figure 5.30 illustrates a tibial section after 20 months of burial. As with the sample buried for 17 months (figure 5.29) the surface of the bone was dark in colour (figure 5.30). There was some plant material in association although this was limited. The bone surface was in good condition, lacking any erosion or scoring damage. Furthermore, fungal activity was absent. The medullary cavity was full of soil.

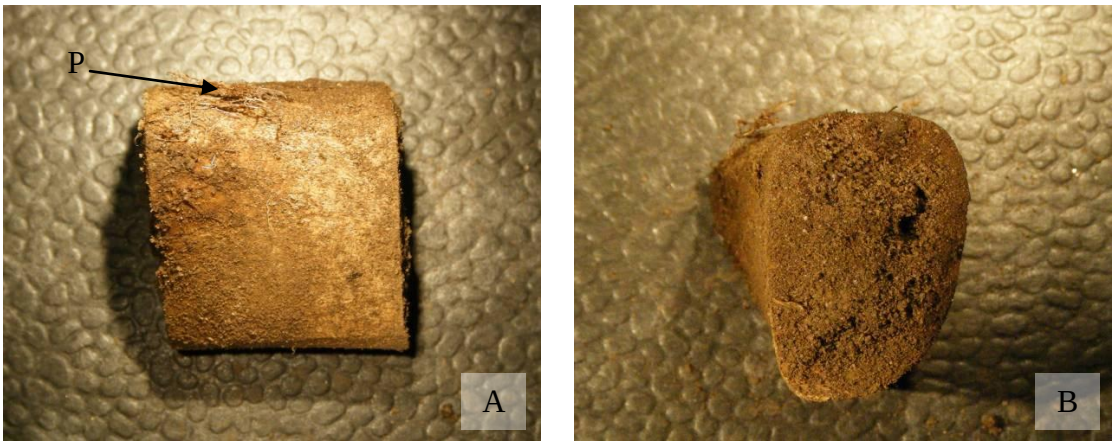

Figure 5.30: Tibia 20 months post burial. A: longitudinal, B: transverse views. P: plant material.

At 22 months burial, the sample exhibited no evidence of degradation, the surface of the bone was smooth in appearance and lacked any erosions or scoring (figure 5.31). The bone surface was dark in colour and minimal plant matter was present. The medullary cavity was partly occluded with soil and no fungi were observed on the sample surface.

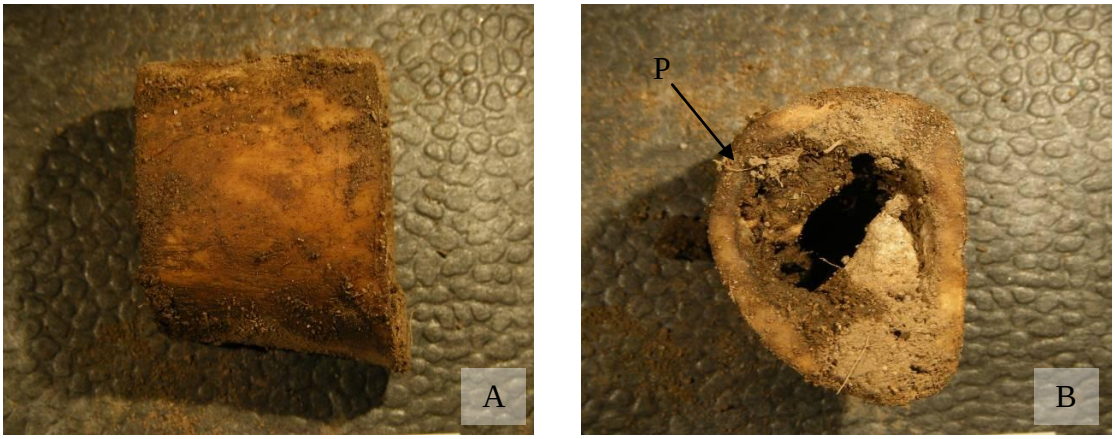

Figure 5.31: Tibia 22 months post burial. A: longitudinal, B: transverse views. P: plant material.

The final sample, at 24 months burial (figure 5.32), showed no macroscopic signs of degradation. The surface was smooth and lacking any erosive changes. There were no fungal structures on this sample. As with the previous samples, there was no bone marrow located within the medullary cavity. However, the cavity was occupied with soil. Small spherical structures were located on the surface of this sample, which were believed to be insect eggs as they were often observed in small clusters. Plant material was absent from this sample.

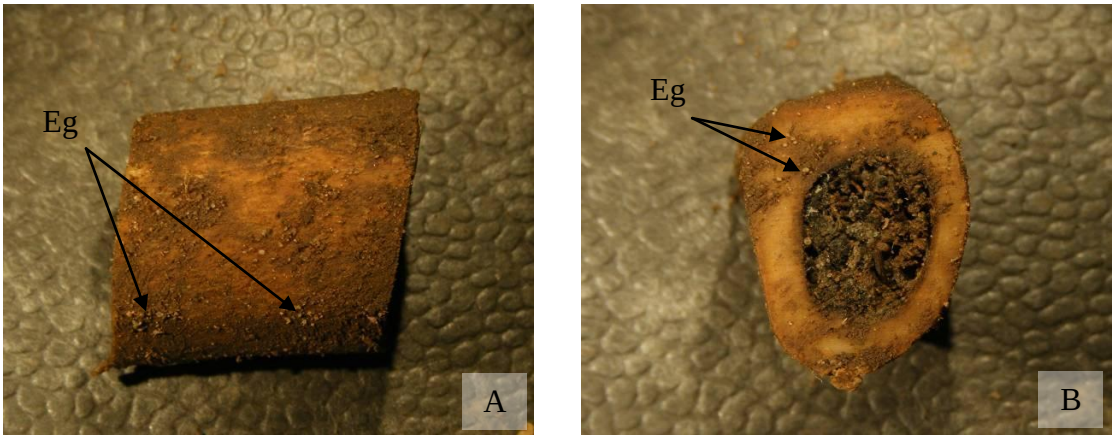

Figure 5.32: Tibia 24 months post burial. A: longitudinal, B: transverse views. Eg: possible insect eggs.

Table 5.2: A summary of the macroscopic data gathered during the postmortem analysis of femur and tibia cross sections.

Sample	Colour	Modification	Marrow present	Colour of marrow	Plant material present	Fungal material present on cortical bone	Soil in medullary cavity
Femur control	I	N	Y	R	N	N	N
Tibia control	I	N	Y	R	N	N	N
Femur 1	I	N	Y	B	Y	N	N
Tibia 1	I	N	Y	W	Y	N	N
Femur 2	I	N	Y	B	N	Y	N
Tibia 2	I	N	Y	W	Y	N	N
Femur 3	I	N	Y	B	Y	Y	N
Tibia 3	I	N	N	N	Y	N	Y
Femur 4	I	Y	Y	W	Y	N	N
Tibia 4	I	N	N	N	N	Y	Y
Femur 5	I	Y	N	N	Y	N	Y
Tibia 5	I	Y	N	N	Y	N	Y
Femur 6	I	N	Y	W	Y	N	Y
Tibia 6	I	Y	N	N	Y	N	Y
Femur 8	I	N	N	N	Y	Y	Y
Tibia 8	I	N	N	N	Y	Y	Y
Femur 10	D	N	N	N	Y	Y	Y
Tibia 10	I	N	N	N	Y	Y	Y
Femur 12	I	Y	N	N	Y	N	Y
Tibia 12	I	N	N	N	Y	Y	N
Femur 14	I	Y	N	N	Y	N	N
Tibia 14	I	N	N	N	Y	N	N
Femur 17	I / D	N	N	N	Y	N	Y
Tibia 17	D	N	N	N	Y	Y	Y
Femur 20	D	N	N	N	Y	N	N
Tibia 20	D	N	N	N	Y	N	Y
Femur 22	D	N	N	N	Y	Y	Y
Tibia 22	D	N	N	N	Y	N	Y
Femur 24	D	Y	N	N	Y	N	Y
Tibia 24	D	N	N	N	N	N	Y

Key: I: ivory, D: dark, Y: present, N: absent, R: red, B: brown, W: white.

Table 5.2 highlights the macroscopic postmortem modifications observed on the femur and tibia cross sections. A change in colour to the cortical bone was noted to occur over time. The femoral bone remained an ivory colour up until eight months of burial. At ten months post

burial the sample had darkened in colour. However, the 12 and 14 month samples were noted to be an ivory colour again. At 17 months post burial the sample exhibited areas of both ivory and dark colouration and the remaining samples (up until 24 months post burial) were all dark in colour. Conversely, the tibia samples were an ivory colour up until 14 months post burial, after which they were all dark in colour until the conclusion of the experiment.

Erosive and degradational modifications were sporadic in occurrence with the samples. The femoral samples did not show any surface modifications until four months post burial. Such modifications were also noted on the five month sample. The six, eight and ten month femoral sections exhibited no surface modifications. At 17, 20 and 22 months post burial degradational changes to the femoral samples was noted. However, the sample buried the longest (24 months) had no erosive changes to the cortical bone. Out of the 14 samples of tibia analysed, only two exhibited post burial changes to the cortical bone (five and six months respectively).

Generally, as the postmortem interval increased bone marrow was lost from the medullary cavity. Bone marrow was present within the medullary cavity of the one, two, three, four and six months femoral samples. All other samples lacked such marrow. The control samples contain red marrow whereas the one, two and three month femoral samples contained marrow which was brown in colour. Furthermore, the four and six months samples contained white marrow. Marrow was recorded in only two of the tibial samples (one and two months post burial). However, this marrow was white in appearance contrary to the femoral bone marrow from the samples with the same burial interval.

Plant material was commonly found on the samples from the earliest burial interval. Such plant material, in the form of roots, was documented on all but the two month femoral sample. The tibial samples exhibited plant growth on all but the four and 24 month samples.

Fungi were noted on the samples but its occurrence was sporadic over the burial intervals. The femoral sections contained fungi, first on the bone marrow, at two and three months post burial. On the eight, ten and 22 months samples, fungi was located on the cortical bone. Fungal growth was observed on the tibial samples, but exclusively on the cortical bone. This growth was documented on the four, eight, 10, 12 and 17 months samples.

Soil infiltration to the medullary cavity was observed as the burial interval increased. However, there were intervals of inconsistency. With the femoral section, soil was absent from the cavity in the one, two, three and four month samples. Interestingly, at 14 and 20 months of burial soil was also absent, but all other samples contained soil in the cavity space.

Similar findings were also documented with the tibial samples. At one and two months post burial soil was absent from the cavity. Soil was also absent in the 12 and 14 months samples. All other tibial samples contained soil within the cavity.

5.3.2 Scanning electron microscopy

Due to the similarities encountered during the SEM analysis of the femoral and tibial samples, it was decided to show one of the representative samples here. The following figures discuss the changes observed to femoral bone over a 24 month period.

Figure 5.31 details the surface of the control femoral section (with no exposure to soil). The image at a low magnification (figure 5.33A) shows that the surface was covered in grainy debris, which was abundant over the entire sample. Due to no exposure to soil, soil artefacts could be eliminated. These grainy structures could be dried residual periosteum adhering to the cortical bone although this could not be determined by this analysis. At a higher magnification the cortical bone could be visualised which was smooth in appearance (figure 5.33B). A foramen was observed in figure 5.33B. No erosions or cracks were observed.

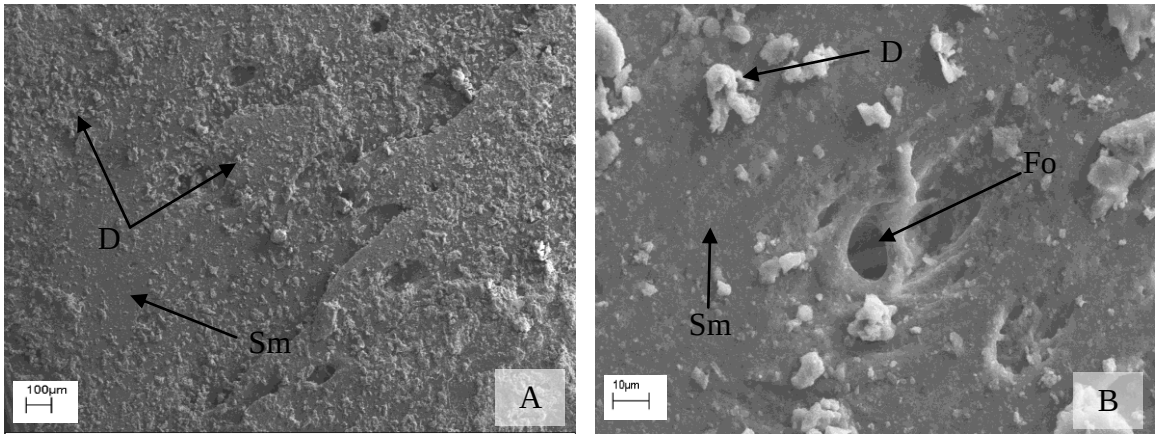

Figure 5.33: Scanning electron micrographs of the control femoral sections. D: debris, Sm: smooth surface, Fo: foramen.

The sample buried for one month appeared to have lost the grainy dried residue over the entire sample. On the higher magnified image (figure 5.34) small debris artefacts were present which could be soil or the dried debris observed in the previous sample (figure 5.33). Overall, the sample exhibited a porous morphology (figure 5.34A). The pores were not a result of post burial modification as they had clear, defined borders. Otherwise, the bones surface was

smooth with no observable erosions or cracks. A foramen was noted (figure 5.34B) which had an unidentifiable structure partly occluding its opening.

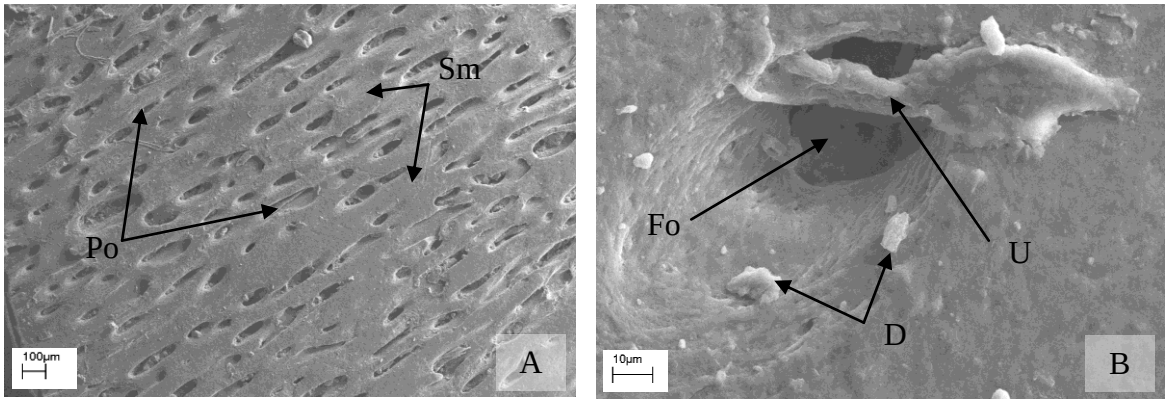

Figure 5.34: Scanning electron micrographs of femoral sections buried for one month. Po: porosity, Sm: smooth surface, Fo: foramen, D: debris and U: unidentified structure.

At two months post burial plant matter was evident on the bone surface (Figure 5.35A) in the form of small rootlets. These were strongly adhered to the cortical bone. Overall the sample was architecturally sound, with no obvious erosive damage. However, the bone was beginning to exhibit some cracking around the foramen as illustrated in figure 5.35B. The debris seen in the previous samples was absent.

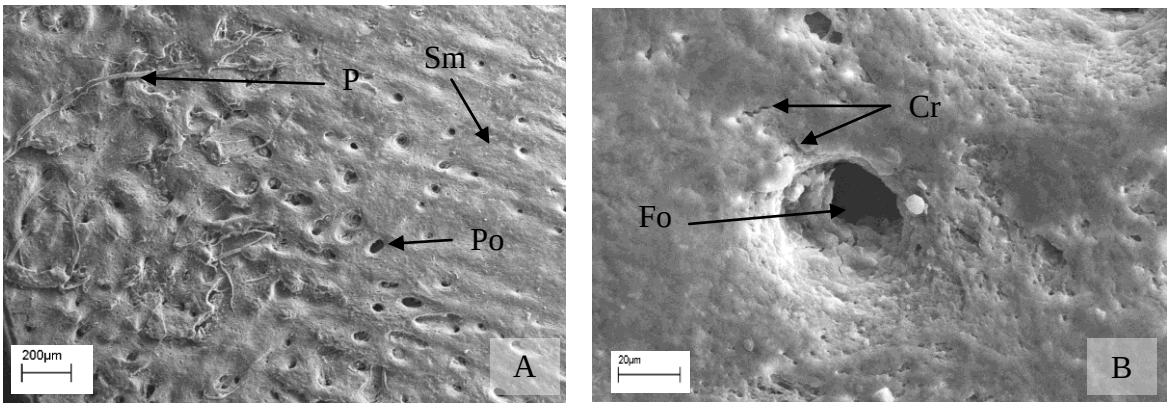

Figure 5.35: Scanning electron micrographs of femoral sections buried for two months. P: plant material, Po: porosity, Sm: smooth surface, Fo: foramen, Cr: cracking.

At three months post burial (figure 5.36) the cracking observed on the two months sample (figure 5.35A and B) had become more extensive and was pronounced on the bones surface. There was no evidence of erosions to the cortical surface as it was smooth in appearance.

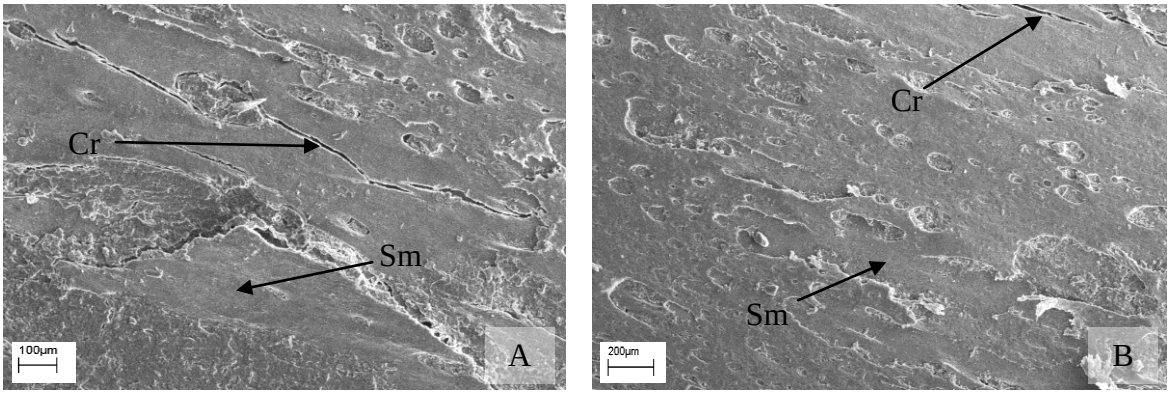

Figure 5.36: Scanning electron micrographs of femoral sections buried for three months. Cr: cracking, Sm: Smooth surface.

Cracks were absent from the sample buried for four months (figure 5.37). However, the cortical bone had begun to show numerous small shallow depressions which may be erosions caused by interment in the burial environment. The possible erosions were widespread on the sample.

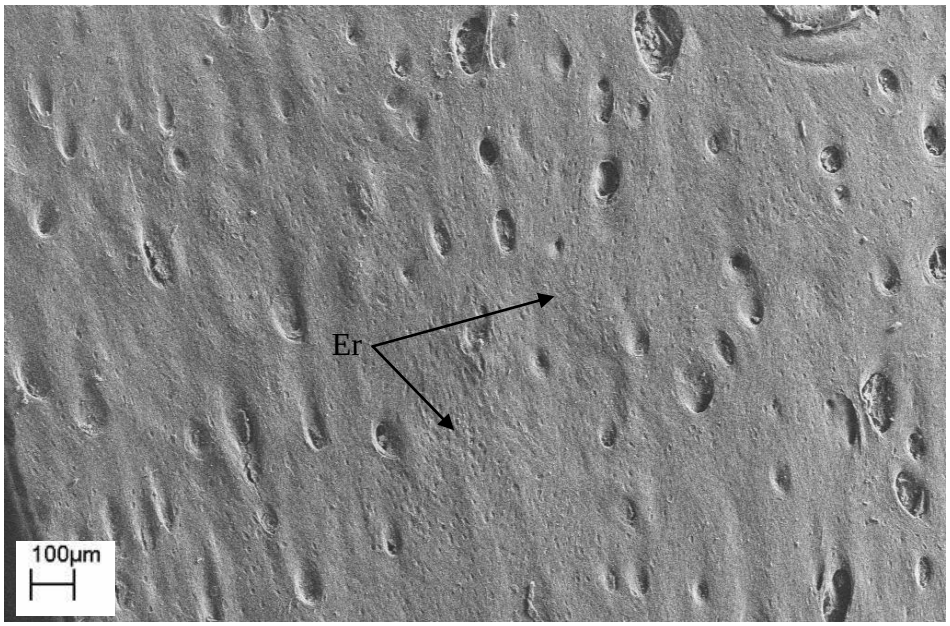

Figure 5.37: Scanning electron micrograph of a femoral section buried for four months. Er: erosions.

The five month sample (figure 5.38) was unlike any previously seen. The smooth surface documented on the other samples (e.g. figures, 5.34, 5.36) had been replaced by a surface that was irregular. The surface of the bone appeared degraded with loss of smooth cortical bone.

The resulting appearance was similar to a trabecular structure. There were plant rootlets present adhering to the bone.

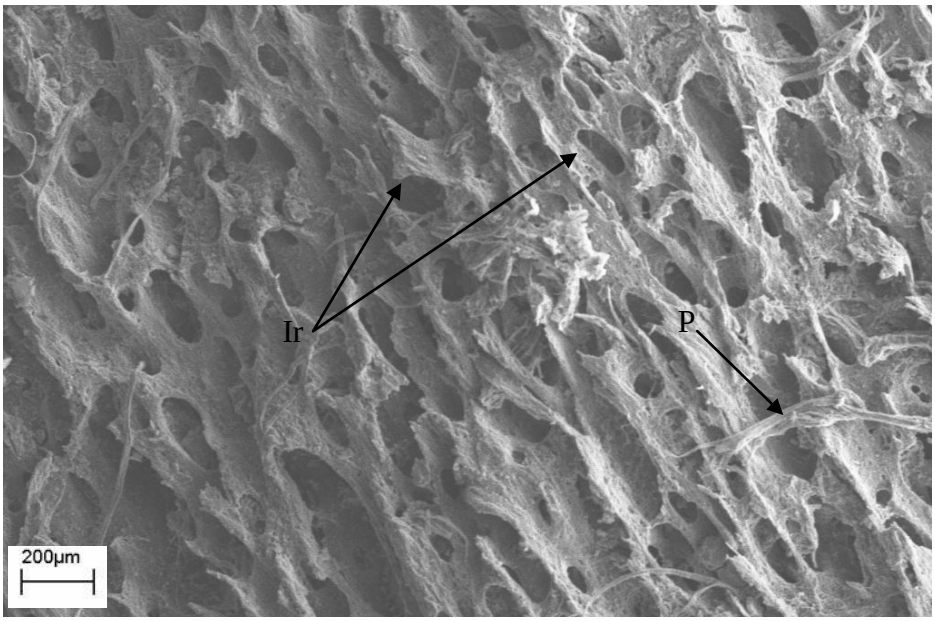

Figure 5.38: Scanning electron micrograph of a femoral section buried for five months. Ir: irregular surface, P: plant material.

At six months post burial (figure 5.39) the bone appeared more degraded than the four month sample but less degraded than the five month sample as the level of degradation appeared in between the morphology of the aforementioned samples. The smooth cortical bone was beginning to degrade with the surface becoming irregular. However, the trabecular appearance seen on the five month sample (figure 5.38) was not observed. Plant rootlets were present on the surface.

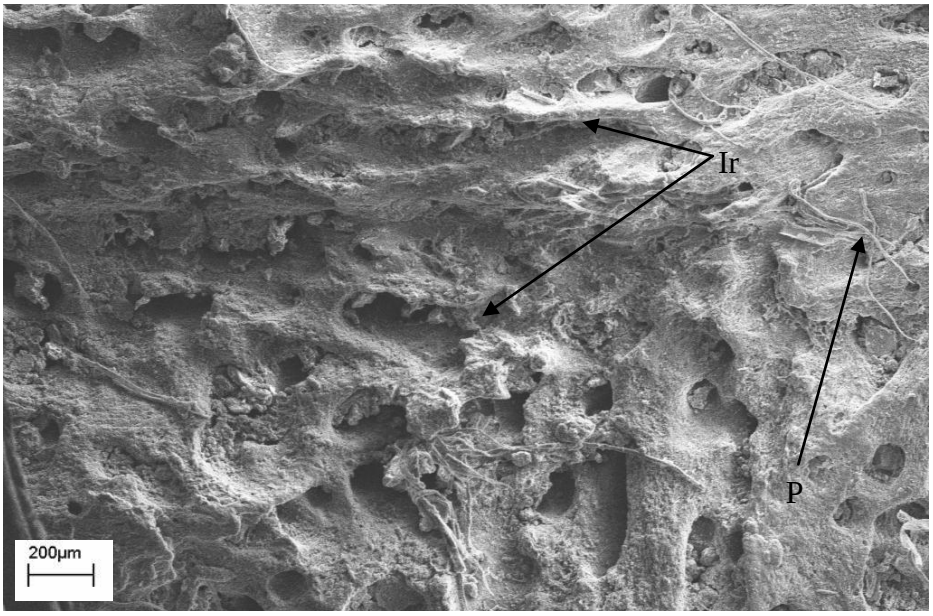

Figure 5.39: Scanning electron micrograph of a femoral section buried for six months. Ir: irregular surface, P: plant material.

At eight months (figure 5.40) the bone appeared in better condition compared to the five and six months samples. Some smooth compact bone was visible but in general the surface was beginning to show areas of irregularity. There was some plant material adhered to the surface of the sample.

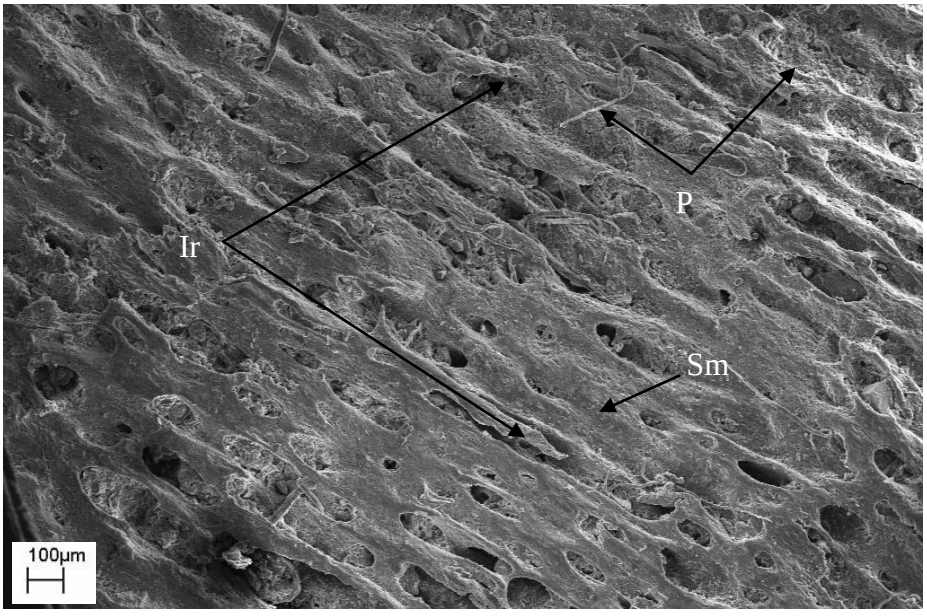

Figure 5.40: Scanning electron micrograph of a femoral section buried for eight months. Ir: irregular surface, P: plant material, Sm: smooth surface.

The 10 months sample (figure 5.41) had some areas of smooth cortical bone although this was limited over the sample. However, the bone surface did exhibit some small erosive depressions. There were cracks manifested on the surface of this sample but these were small in size. Furthermore, there was some plant material present.

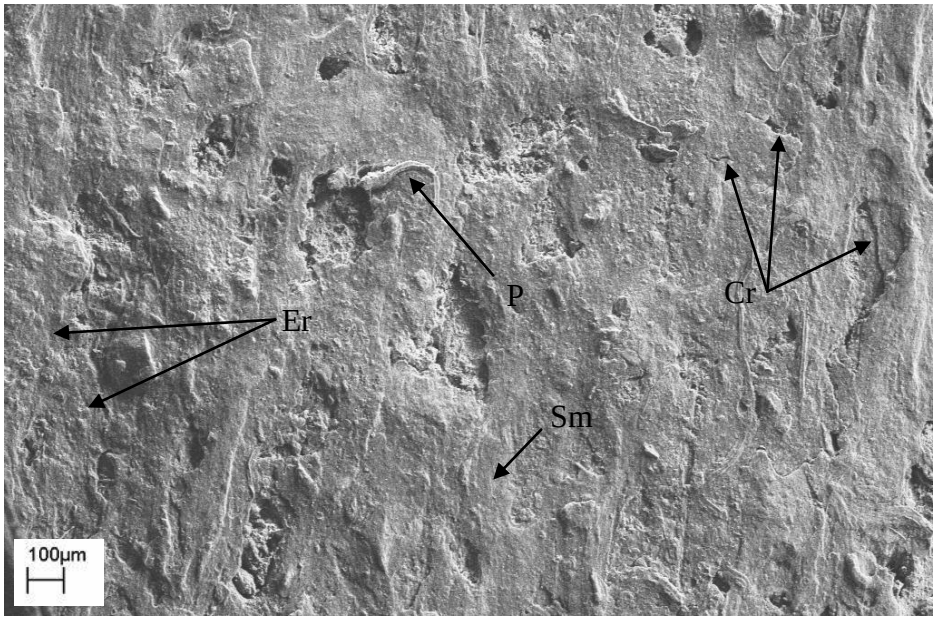

Figure 5.41: Scanning electron micrograph of a femoral section buried for 10 months. Er: erosions, Sm: smooth surface, P: plant material, Cr: cracking.

At 12 months post burial (figure 5.42), the bone surface exhibited some flaking. This flaking was localised and not widespread on the sample. The bone was absent of any cracking. However, there was a possible area of extensive erosion. This area was observed as an area of depression in the surface, which was porous in appearance. Smaller areas of erosive damage to the surface were also noted.

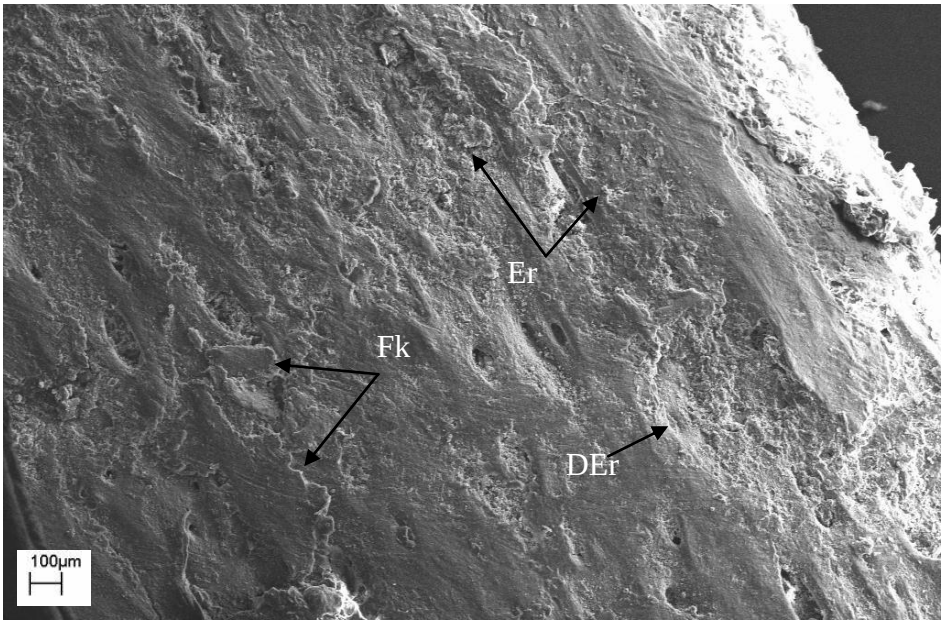

Figure 5.42: Scanning electron micrograph of a femoral section buried for 12 months. Fk: flaking of cortical bone, Er: erosions, DEr: depressed area with erosions.

Figure 5.43 details a femoral bone sample at 14 months post burial. There was some plant material in the form of roots on the surface. The scoring present on the top right of the sample was caused by a circular saw during sample preparation and was not related to post burial damage. There was no cracking damage manifested on this sample but there was a possible area of flaking. The image was darker in comparison to previous, due to malfunction of the SEM and the utilisation of a different detector. However, the resulting image was suitable for this analysis.

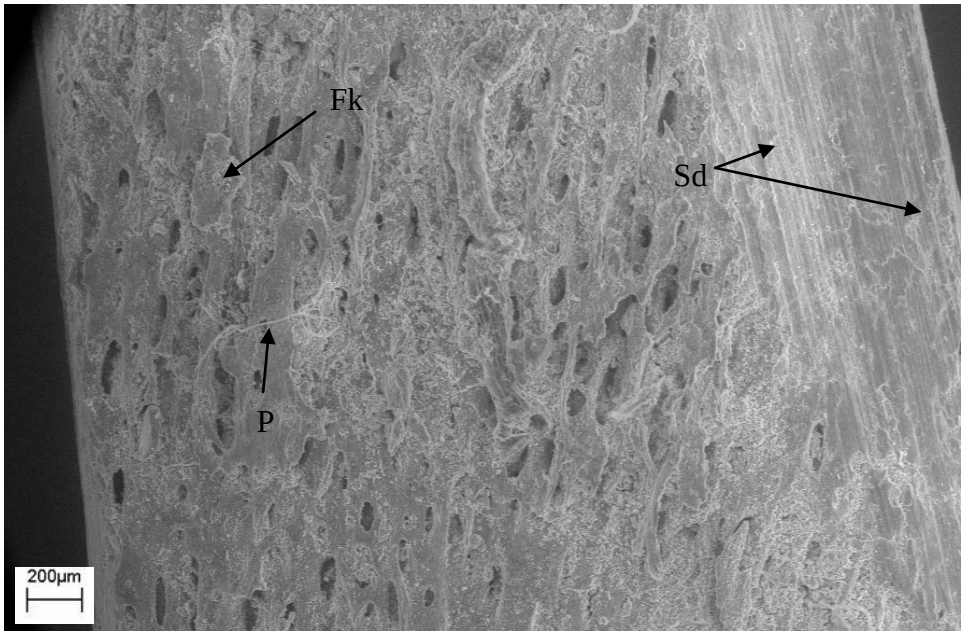

Figure 5.43: Scanning electron micrograph of a femoral section buried for 14 months. P: plant material, Fk: flaking, Sd: sawing damage.

Cracking to the bone surface was documented again on the sample buried for 17 months (figure 5.44). The cracking was apparent across the entire sample. Furthermore, a possible area of flaking was noted on the surface. The cortical bone had regions of a smooth appearance but it also manifested irregular and grainy areas. The darker image was due to using a different detector.

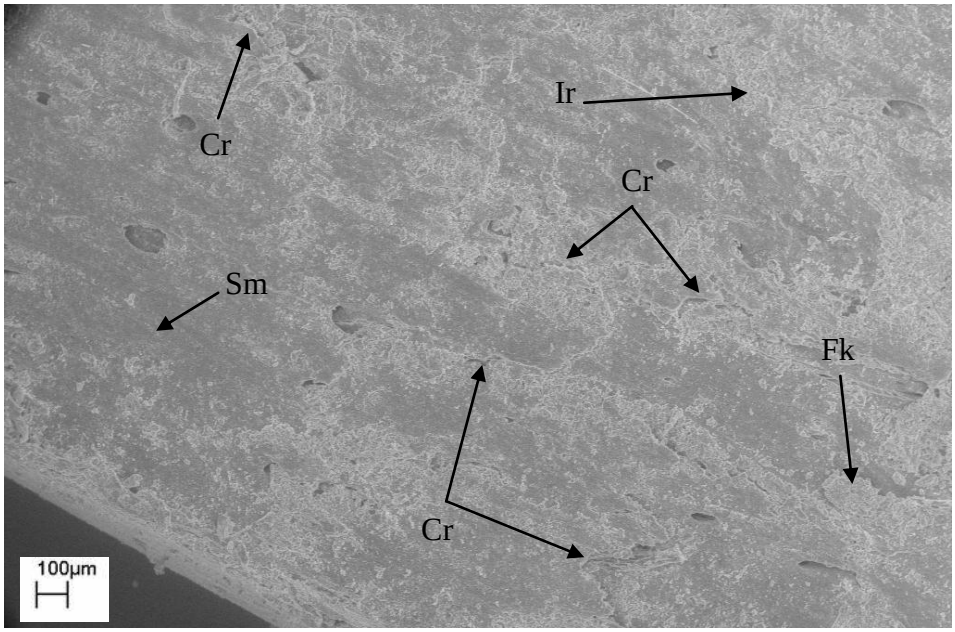

Figure 5.44: Scanning electron micrograph of a femoral section buried for 17 months. Cr: cracking, Fk: flaking, Ir: irregular / grainy appearance, Sm: smooth appearance.

The sample interred for 20 months exhibited pronounced cracking to the bone surface as illustrated in figure 5.45. The cracking was widespread on the sample. There was no plant material in association. The surface exhibited an irregular and grainy appearance. The image was darker due to utilisation of a different detector.

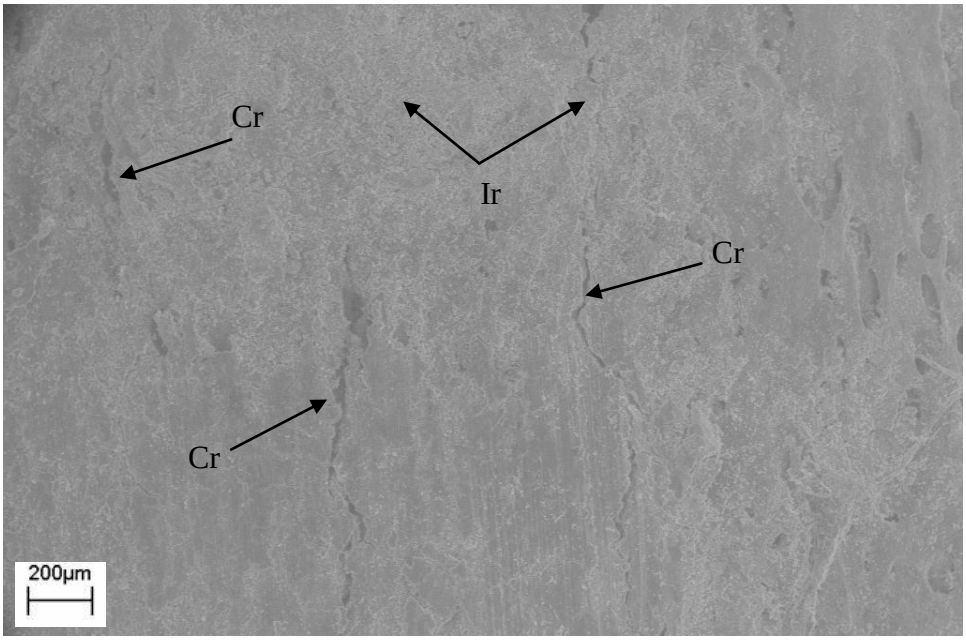

Figure 5.45: Scanning electron micrograph of a femoral section buried for 20 months. Cr: cracking, Ir: irregular / graining surface.

Widespread cracking was a prominent feature on the sample buried for 22 months (figure 5.46). Associated plant material was absent from the surface of the sample. An area of flaking cortical bone was observed, but this was localised and not widespread. Areas of smooth cortical bone were noted, but areas of a grainy and irregular appearance were also observed.

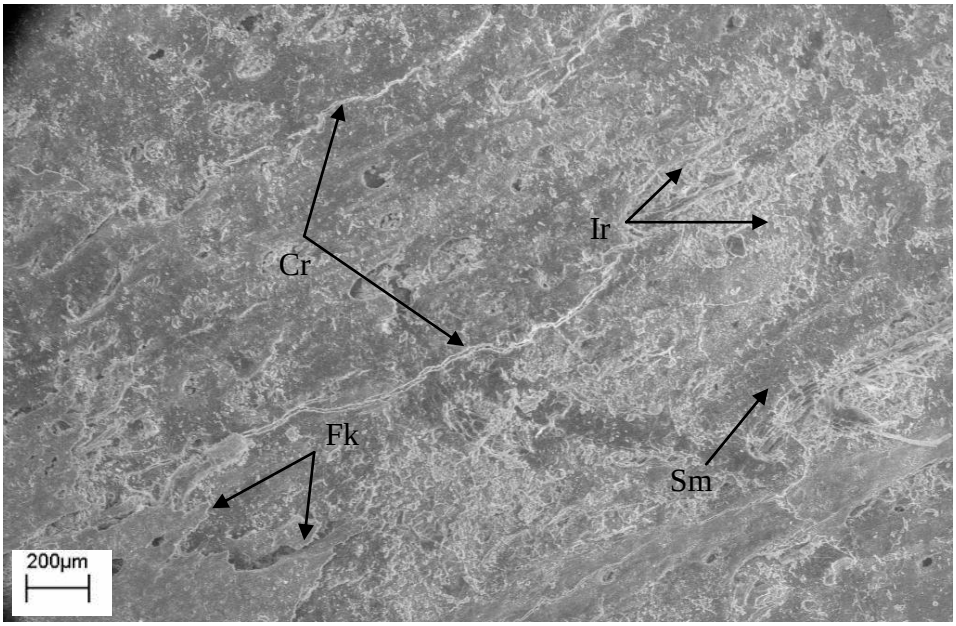

Figure 5.46: Scanning electron micrograph of a femoral section buried for 22 months. Cr: cracking, Ir: irregular / grainy appearance. Fk: flaking, Sm: smooth appearance.

The final sample, interred for 24 months (figure 5.47), exhibited cracking on the bone surface. These cracks were prominent in size and were widespread over the sample. The sample surface exhibited areas of a smooth texture, but there were also areas which were grainy in appearance. Regions of cortical flaking were not documented.

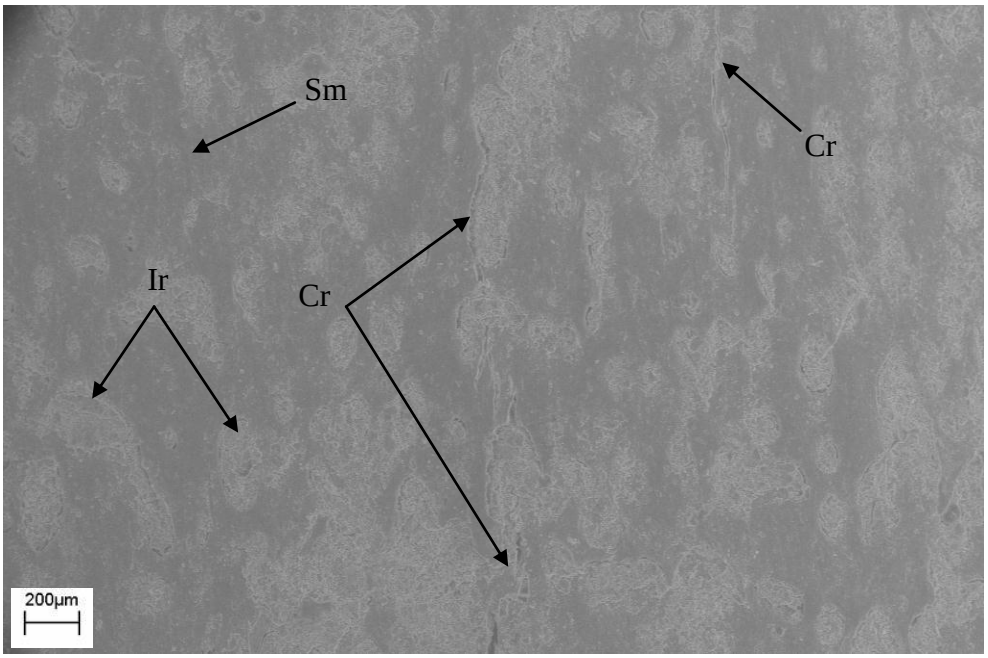

Figure 5.47: Scanning electron micrograph of a femoral section buried for 24 months. Cr: cracking, Ir: irregular / grainy appearance, Sm: smooth appearance.

5.4 Discussion

Macroscopically, postmortem changes to the bone were observed to be inconsistent over the 24 months of burial. Such postmortem changes were subtle and limited to changes in colour, possible erosions to the cortical bone, and colonisation by fungi. Erosions to the bone surface were first noted at four and five months after burial with the femoral and tibial samples respectively (figure 5.7 and 5.23). However, such erosive modifications were not consistently observed. As the experiment progressed, some later post burial samples exhibited smooth, uneroded cortical bone (e.g. the femur and tibia samples after 17 months of burial: figure 5.14 and 5.29 respectively). The erosions were manifested as areas of bone which were grainy and irregular in texture. On occasion (e.g. femur after five months of burial: figure 5.8), score like marks were present on the sample. These erosions could be due to a detrimental interaction between the bone and soil, which was found to be acidic within the grave.

Fungi were found to colonise the residual bone marrow contained within the femoral samples at two and three months post burial (figure 5.5 and 5.6). However, no fungi were observed on the tibial bone marrow. There is no known literature that details fungal growth on bone marrow and this is believed to be the first documented case. Whilst the majority of human remains are intact upon interment, fungal growth on bone marrow could possibly yield information relating to time of death in cases of dismemberment if fungal colonisation follows a predictable succession. However, bone marrow was found to degrade quickly at Compton (within 6 months) indicating a short window of opportunity for analysis. This aspect certainly warrants further detailed analysis. Interestingly, a fungus of a different morphology was present on the bone surface over a 22 month period, (femur samples: eight, 10 and 22 months and tibia samples: four, eight, 10, 12, and 17 months). This observation of fungi on the bone surface is supported by Wedl (1864), Piepenbrink (1986) and Hawksworth and Wiltshire (2010). In addition, in most instances, as the bone marrow was lost, soil began to infiltrate into the medullary cavity, suggesting that soil is a dynamic moving environment (e.g. Balek, 2002).

Plant material was found in direct association with the bone throughout the 24 months of the experiment. In some instances, for example, the femoral sample at four months post burial, the plant growth was situated around the transverse section near the remaining bone marrow. This finding suggests that the plant matter is assimilating the nutrients released during decomposition of the bone marrow. The growing plant material could be responsible for some

of the pitting / erosions manifested on the bone surface due to the release of acids during plant metabolism (e.g. Schultz, 1997 and Harington, 2002).

Entomological activity was found in association with the bone samples in the early stage of burial (the femoral sample at two months post burial: figure 5.5). This material was observed to be pupal cases, possibly from an insect species feeding on any residual soft tissues left adhering to the bone.

Changes in colouration, from ivory to brown, occurred at 10 months (femur) and 17 months (tibia) after burial. This correlates with the literature detailing colour changes to bone in acidic environments due to transitional metals and humic acids becoming freely available (Turner-Walker, 2008).

Analysis with SEM of both the tibial and femoral samples yielded similar manifestations. On the control sample a powdery residue was observed. This was documented as numerous small clusters adhering to the entire cortical surface. As the control bone was not buried, soil artefacts could be eliminated as the causative agent. Furthermore, the control bone was defleshed, sectioned and frozen until analysis, thus one possible explanation of these artefacts could be dried residual periosteum. However, this could not be confirmed based on this analysis. This dried residue was absent by the second month of burial leaving a smooth osteological surface. At two months post burial small localised cracks were noted around the foramen and plant rootlets began to colonise the surface. These were not observed during macroscopic analysis. Cracking manifested on the bone surface was pronounced on the three month sample and observed again at 17, 20, 22 and 24 months post burial. The cause of such microcracks could not be conclusively determined, but published literature highlight causative agents including remineralisation due to incorporation of ions or due to microbial activity (Piepenbrink, 1989, Grupe, Dreses-Werringloer, 1993, Child, 1995), the loss of organic matter resulting in shrinkage (Piepenbrink, 1986) or exposure to high temperatures (Holden *et al.*, 1995). However, the latter factor could be disregarded due to burial within a soil environment. Cracking due to burial pressure could also be dismissed due to the shallow nature of the graves. The microcracking manifested consistently at the end of the experimentation (months 17-24) but the presence of cracking at three months warrants caution when assessing the presence of this phenomenon to determine a postmortem interval. Possible cortical flaking was also observed on the samples from 12, 14, 17 and 22 months post burial. This was visualised as small regions of flat bone partly lifted from the sample surface. Other surface features included areas of irregularity. This factor was especially pronounced on the

five month's sample (figure 5.38) whereby the bone had lost its smooth cortical appearance and resembled disorganised trabeculae. Small shallow depressions on the cortical bone were noted on some samples (e.g. four month: figure 5.37). These depressions could be a result of modification in the burial environment although this, at present, is speculation.

The general degradation observed to the bone samples over 24 months was not consistent, nor followed a predictable pattern. The colour change was the only factor which exhibited a gradual progression over time. However, as stated previously, this could be due to the acidic nature of the soil and may not be as consistent in other soil environments. Bone marrow was present up until six months post burial in the femur samples but was absent in the five month's sample. Furthermore, the tibia samples only contained marrow until three months. Additionally, the colour change to the marrow (brown in the femur, and white in the tibia) was not consistent between the two sample types. The observation of pitting and depressions, flaking, and cracking were found to be inconstant over time.

Unfortunately, due to a lack of a suitable microtome, and despite sending bone samples away to be sectioned. The desired histological sections were not obtained within the time dedicated to this study.

Based on this analysis using macro and microscopic methods, the post depositional modifications to cross sections of tibia and femur were of very limited value for determining an estimate of the postmortem interval.

Chapter 6: Decomposition of Whole Piglet Carcasses

"I see no more than you, but I have trained myself to notice what I see".

Sherlock Holmes, *The Adventure of the Blanched Soldier*.

6.1 Whole Pig Corpses

Pigs (*Sus scrofa*) are widely used in taphonomic experiments as a model for human remains (Shean *et al.,* 1993, Turner and Wiltshire 1999, Davis and Goff 2000, Weitzel, 2005, Matoba, 2007, Wilson *et al.,* 2007). Pigs have intestinal flora similar to humans, and share similar skin morphology (Campobasso *et al.,* 2001). Within the published studies pigs were utilised to represent small to large sized human cadavers (Turner and Wiltshire 1998, Wilson and Janaway *et al.,* 2007). Galloway *et al.,* (1997) suggest that pigs used should be healthy and young in age as in later life bone mineral density decreases which has an effect on the preservation of bone.

The time taken for skeletonisation can vary remarkably. Weitzel (2005), conducted experiments on pigs subjected to different conditions (clothed, unclothed, covered in sediment, covered in stones, burnt, wrapped in birch bark, covered in red ochre and buried at different depths). There was a difference in the levels of decomposition between the samples with only some of the samples reaching skeletonisation within 15 months. Additionally, Prieto, Magana and Ubelaker (2004), report skeletonisation in bodies that had been interred for a period of 2.5 years, and Rodriguez and Bass (1985), documenting skeletonisation only occurring to the hands and feet after one year of burial at a depth of four feet.

6.2 Methods

6.2.1 Burial set up

Three deceased piglets were obtained from a local farm (figure 6.1). The piglets were one to two weeks old at the time of death. The specimens were obtained less than 24 hours after death. Death was a result of trampling by their mother. The piglets were intact with no wounds present on the dermis and the mucus membranes were still wet. The maximum and minimum lengths for the piglets were 18 and 14.5 inches respectively. The objective of this analysis was to document the time taken until exposure of the skeleton in Compton campus soil. This data could then be applied to the changes observed during the bone experiments (chapter 5) as a correction factor. Furthermore, such data could be applied to the trotter degradation to determine if amputation influenced the decomposition process.

Burial occurred in a fenced pen located at Compton campus. Each piglet was interred in a single grave to a depth of 20 cm. A gap (1.5 ft) was left between the graves to avoid any cross

contamination between the samples or prevent a passageway for macro / micro fauna. Furthermore, each pig, and thus grave, was excavated for analysis once only.

The burial complied with the regulations set by the Department for Environment, Food and Rural Affairs, Leicester Animal Health Divisional Office, Leicester (Appendix one).

Figure 6.1: The piglets used for the interment. All were approximately the same size and had similar postmortem intervals of approximately 24 hours. Death was due to natural causes.

6.2.2. Sample analysis

The excavation of samples occurred at three, six and eight months respectively. Small layers of soil were removed to prevent unnecessary damage to the sample (Hunter and Dockrill, 1996). Prior to excavation, the ground surface on and around the grave site was analysed to document the presence of any fruiting fungi. The stage of decomposition was documented using a digital camera (Coolpix 4500, Nikon).

6.3 Results

Out of the three samples analysed, only the three and eight month sample have been detailed here.

6.3.1 three months post burial

The area of ground above the grave exhibited prolific floral growth greater than that of the surrounding area (figure 6.2). This abundance of flora is consistent with the current published literature stating that the growth of vegetation situated above a grave would increase due to release of nutrients during decomposition (Bass and Birkby 1978, Rodriguez and Bass, 1985, Owsley, 1995, and, Nawrocki, 1996).

Figure 6.2: The excessive vegetation located over the grave site. The arrow shows grave markers situated in the upper left corner of each grave.

When the grave was excavated, the carcass was still intact with abundant soft tissue in association (figure 6.3). The skin was predominantly black in colour, consistent with putrefactive changes. The skin was fragile and cleaning of the carcass for photodocumentation resulted in skin slippage exposing pink musculature. Due to this ease of damage an excavation of the entire carcass was not carried out. However, the entire thorax and abdomen was excavated indicating that preservation was good. Accidental puncture of the abdominal cavity resulted in a release of malodorous gases. Palpation of a small area of the abdominal

cavity resulted in movement of the thorax caused by putrefactive gases contained within the body cavities.

Areas of the outer extremities, namely the legs, had begun to skeletonise at the distal aspects (the trotters). This was consistent with the level of decomposition seen in amputated trotters at this stage of decomposition (Chapter 3.3.1). Maggots were absent from the carcass. Earthworms were present in abundance. Fungal growth was not present around the grave.

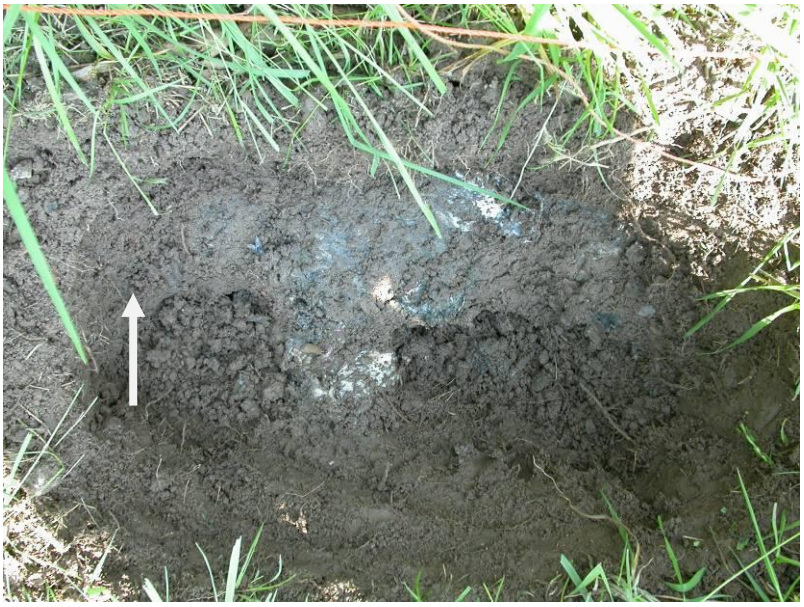

Figure 6.3: The decomposition of the carcass 3 months post burial.
The arrow marks the head.

Exposure of the carcass to the open environment was kept to a minimum to prevent accelerated decomposition from exposure to atmospheric oxygen. Within a few minutes, flies, from the order Calliphoridae, began to appear. After photodocumentation was complete the carcass was reburied to allow for further analysis in the future, if required.

6.3.2 eight months post burial

The eight months' sample had decomposed remarkably (figure 6.4). The majority of the corpse had skeletonised. There was still some soft tissue in association (in the form of white masses). However, this was minimal and only adhered to isolated skeletal elements. Numerous bones were visible including, ribs and limb long bones. The cranial bones were moved as a consequence of the excavation process. The cranial bones were not fused due to the pig being an immature individual. This result indicates that it took less than eight months for a corpse to exhibit extensive exposure of the skeleton in Compton soil. Plant growth was prominent over the grave. Earthworms were observed in close association with this sample.

Figure 6.4: The decomposition of the carcass eight months post burial.

6.4 Discussion

Whole piglet carcasses (figure 6.1) were buried with the aim to document the time taken for exposure of the skeleton in Compton campus soil. The samples progressed through the typical decomposition processes with evidence of skin slippage and putrefaction documented at three months after burial (figure 6.3). Interestingly, exposure of the skeletal elements of the trotters occurred within three months concurring with the findings for the amputated trotters (Chapter, 3.3.1). This suggested that amputation did not influence the decomposition process significantly. The time taken to reach exposure for the majority of the skeleton was eight

months (figure 6.4). This was less than the time intervals presented by Weitzel (2005), Prieto, Magana and Ubelaker (2004), and Rodriguez and Bass (1985), most probably due to environment conditions and the depth and characteristics of the burial. Prolific floral activity was noted around the grave (figure 6.2) concurring with the literature (e.g. Bass and Birkby 1978, Rodriguez and Bass, 1985, Owsley, 1995, and, Nawrocki, 1996). However, after several years, beyond the analytical experimental part of this study, no fungal growth had been observed around the grave sites thus differing from the findings of Carter and Tibbett (2003).

The young age of these piglets could have impacted on the decompositional processes, other literature utilised pigs of a much larger weight (e.g. Turner and Wiltshire, 1999). But, due to space and cost issues, large pigs were impractical for this analysis. For future experimentation detailing the specifics of whole cadaver decomposition, a grading system (e.g. Galloway *et al.*, 1989) should be used.

Chapter 7: General Discussion

"Science, in the very act of solving problems, creates more of them".

Abraham Flexner 1930

Of the three tissues analysed it was found that all exhibited degradation over time. The overall time frame for the degradation was variable depending on the density, composition of the tissues and the characteristics of the burial environment.

The first tissue to completely decompose was cartilage, which was usually decomposed and absent from articular facets after 12 to 13 weeks postmortem (figure 3.14). The trotters which contained the cartilage exhibited a progressive degradation with skin slippage (figure 3.8) and liquefaction (figure 3.12). These characteristics are part of the decomposition process, whereby preservation has not occurred (e.g. Clark *et al.,* 1997 and Knight, 2004). The cartilage, as an individual tissue, exhibited a characteristic decomposition process. Macroscopically, a change in cartilage colour was noted. This was observed to be a cream white colour in the control tissue (figure 3.5) to pink (figure 3.8) and finally to a dull cream colour (figure 3.14D) towards the end of the cartilage decomposition process. The macroscopic findings such as the colour changes, amount of cartilage covering the articular facet and cartilage texture allowed for the adaptation of a grading system presented by Fuller *et al*., (2002). This system allowed for the level of degradation of cartilage to be assigned a score of 0 to 4, with 0 being undamaged to 4 indicating a severe loss of cartilage.

Histological sectioning and staining of the cartilage with H&E was successful with a loss of nuclei over time. This data was consistent with the findings presented by Lasczkowski *et al.,* (2002). Unfortunately, the application of a further histological stain (Titan yellow) to test for the presence of magnesium was inconsistent (chapter 3.3.7).

SEM analysis showed that orthorhombic crystals formed on the cartilage surface over time (figure 3.16). The crystals were found on the surface from three to six weeks postmortem. SEM-EDX analysis showed that the five major elements within the crystal structure were carbon, nitrogen, oxygen, magnesium and phosphorus (chapter 3.3.5). These crystals were found to form outside of a soil environment, indicating that the soil is not a influencing factor for their development (chapter 3.3.6) A pH change to the cartilage surface (pH 7 to 8) was noted to correlate with the precipitation of these crystals (chapter 3.3.9). Microbiological experiments (chapter 3.3.3.1 and 3.3.4) strongly suggested that bacteria were the causative agents for the crystal precipitation. *Acinetobacter calcoaceticus, Acinetobacter iwoffii* and *Grimontia hollisae* were presumptively identified based on GC-MS analysis of their fatty acids. Furthermore, the crystal producing bacteria was suggested as a *Comamonas* species. Until now these bacteria remain unreported on cartilage postmortem.

The application of XRD analysis, coupled with the SEM-EDX data, and consultation with relevant publications (Scudder, 1928, Rivadeneyra *et al.*, 1992, Rivadeneyra *et al.*, 1993, Doyle and Parsons, 2002, Lee *et al.*, 2009) suggested that these crystals were consistent with struvite. Successful application of the cartilage methods to other animal species, (chapter 3.3.11) indicated that the crystal formation observed was not limited to porcine samples.

Human head hair was found to persist longer than cartilage in a burial environment. Depending on the burial conditions, it was noted that hair could be in an advanced stage of degradation, with breakdown of the hair shaft in as little as 15 weeks (figure 4.6). However, it was also noted to be in good general condition after 24 weeks in some instances (figure 4.5). When exposed to a decomposing meat source, it was observed that hair preservation was good compared to when buried without such exposure (compare figure 4.4 with figure 4.5, and figure 4.6 with 4.7). This finding correlates with the current publications (Wilson, 2008). Damage to hair within a burial environment was observed as a loss of cuticle scales (e.g. figure 4.4F), erosions and breaks to the hair shaft (figure 4.4F and 4.6C) and tunnelling (figure 4.8). This study provided strong evidence that fungi could be the causative agent for transverse tunnels observed in hair shafts (chapter 4.3.7). However, it was found that not all of the fungal isolates collected were able to form such tunnels. This study also presents unique data for the type of fungi, and also the morphology of fungal spores observed on the hair shaft (4.11 and figure 4.10).

DNA analysis based on the internally transcribed spacer region (ITS) allowed for the identification of some of the fungal species collected. Unfortunately, only two species could be presumptively identified, *Aspergillus fumigatus* and *Penicillium* (*chrysogenum*). This research provided evidence to suggest that *Penicillium* is able to cause the fungal tunnelling observed in figure 4.8.

In contrast to the cartilage and hair samples, bone exhibited very little degradation after two years in the burial environment (figure 5.17 and 5.32). The cortical bone modifications observed included a change in colour from ivory to dark brown (e.g. figure 5.4 and figure 5.17), erosions to the cortical surface (e.g. figure 5.24) and a colonisation by fungi (e.g. figure 5.10). Bone marrow contained within the medullary cavity was found to degrade over time, and in some instances (e.g. figure 5.5), it was colonised by fungi. It is believed that this is the first documented instance of fungi colonising bone marrow postmortem.

SEM analysis of tibia and femoral bone sections yielded similar results. Post burial modifications included, irregular grainy textures (figure 5.39), possible erosions (figure 5.37),

an apparent loss of surface cortical bone (figure 5.38), possible flaking of bone (figure 5.42) and cracking (e.g. 5.46). Unfortunately, histological sections of the buried samples could not be obtained during this study.

Entire porcine carcasses exhibited soft tissue decomposition at three months of burial with skin slippage and putrefaction observed (figure 6.3). After eight months of burial, most of the soft tissue was absent with only small residual, unrecognisable, areas left adhering to isolated bones (figure 6.4). The skeletonisation of the whole piglet trotters correlated with the time taken for loss of tissue from the amputated trotters used in cartilage experiments (chapter 3). This suggested that amputation of the trotters did not significantly influence the decomposition process. Prolific plant growth was documented over the grave site (figure 6.2) correlating with previous publications (Bass and Birkby 1978, Rodriguez and Bass, 1985, Owsley, 1995 and Nawrocki, 1996).

Most soft tissues were lost from the whole piglets after eight months of burial with exposure of the majority of the skeleton. This data was taken as a correction factor for the bone experiments. Therefore, for any bone modifications, such as pitting or microfractures, the eight month interval can be added to give an overall probable time length.

Overall, cartilage remains the most interesting and promising aspect of this study. The degradation observed was consistent and reproducible in all experiments conducted. The presented findings could provide the basis for a new method for determining the postmortem interval of buried human remains. Unfortunately, the degradation of hair and bone proved to be too variable and inconsistent to be of any value for determining the postmortem interval. Although there was a noted increase in colonisation of fungi on the hair samples over time (figure 4.2), it is probably impractical if not impossible to standardise this at the present time. The dynamics of fungi in the soil environment needs to be fully understood before fungal colonisation on any tissue can be used to determine an estimate of the postmortem interval. In contrast to the bone and hair samples, cartilage remains compartmentalised within the trotter and is not directly subjected to the soil environment. This could explain why cartilage is a valuable and promising tissue for future application in forensic science.

The irregular and inconsistent degradation to hair and bone could be due to the loss, and lack of any protective surrounding soft tissues. The bone was interred absent of most of the overlaying soft tissue (defleshed) and thus directly subject to soil contact. Furthermore, hair has no protective covering and is exposed to soil in a burial situation.

When research is to be conducted on tissues in direct contact with soil (i.e. hair or defleshed bone) then a thorough understanding of the soil environment is required. If a burial environment is reused on several occasions then established faunal saprophytic communities may be present, thus resulting in inconsistent findings on behalf of the researcher. This is highlighted by Wilson *et al.*, (2007) who explains that in the context of burial the decomposing pig carcass will provide nutrients, similar to those found in laboratory media, thus leading to higher microbial counts.

At present, due to our limited knowledge of the soil and its impact on decomposition, tissue which remains internal to the subject for as long as possible during the decomposition process, such as cartilage, remains a possible hope for developing methods for estimating the postmortem interval of buried remains.

This study has highlighted the need for a multidisciplinary approach when studying taphonomic phenomena. This approach will ensure that biotaphonomic and geotaphonomic aspects are considered. When a decomposing corpse is interred, the grave's environment will influence the progression of the decomposition process. Furthermore, the presence of decomposing remains will influence the characteristics of the soil within the grave (see Nawrocki, 1996).

Initially, it was thought that the amputation site on the trotters could act to accelerate the decomposition process. However, the experiment on whole porcine carcasses has partly eliminated this concern.

The successful application of the cartilage methods to other animal species has provided evidence that such processes are not limited to porcine samples. Furthermore, the successful application of the cartilage methods to other animal species may provide a PMI method for application in wildlife forensics. To determine, for example, if an animal was killed outside of legitimate hunting seasons. Ideally, this method should be applied to human remains to determine if crystal formation occurs. However, at present this is not likely to occur in the United Kingdom due to the Human Tissue Licence and its restrictions along with societies overall view on the treatment of deceased individuals.

The main limitation of this study is the utilisation of only one soil type. Future work should be undertaken at sites with different soil characteristics such as an alkaline pH to determine the true range of difference in other burial locations. Further work to determine the specific bacteria (by DNA analysis of all isolates) along with their origin and how they enter the joint space is paramount. Additional work should focus on specifically identifying the *Comamonas*

species. Also, to further document the effect of this bacterium on cartilage, sterile cartilage should be inoculated with a pure strain. This will allow for the observation of possible crystal precipitation in a non-competitive environment (with other bacterial species absent). Such experiments should be conducted using all of the bacteria identified. Additionally, more analysis (in the form of XRD) must be conducted to determine a definitive identification of the crystal species, possibly by utilising the expertise of a crystallographic expert. The change in pH observed to the cartilage surface should also be investigated further. A pH method with a narrow focus (compared to the pH 1-11 litmus paper used during this study) may produce more specific pH data that may illustrate a narrower range for precipitation of the postmortem crystals. It is also of great importance to see if different soil types will alter the 22-25 day time interval (observed during this study) for pH changes to the cartilage surface. As crystals were seen forming on the cow and goat samples, further work should be focused on determining when the precipitation occurs and if this correlates with the crystal precipitation documented in the porcine samples.

Further experiments investigating the fungal colonisation on hair should focus on accurate fungal identification based on DNA sequencing. This eliminates the possible misidentification by morphological analysis by persons with limited experience in fungal taxonomy (Bunyard, 2004). The sequences obtained during the course of this research should be further scrutinised to allow for definitive identifications using BLAST. Further investigation should also focus on the interaction of a decomposing meat source and fungal growth, and why, under such conditions, hair is better preserved. The incidence of tunnelling caused by the possible *Penicillium chrysogenum* should be further investigated. Sterile human hair should be inoculated with a known culture of *Penicillium chrysogenum* and analysed to document the formation of transverse tunnelling.

Cross section histological analysis of the buried bone samples should be undertaken to determine any post burial modifications within the bone micro structure. However, it is possible that such modifications will be as inconsistent as the surface changes observed. It would be of interest to determine if the fungi growing on the cortical bone were able to penetrate and tunnel into the bone structure.

This study by its nature, as a novel piece of work, is largely descriptive in its approach. However, this was unavoidable as, especially relating to the cartilage aspect, previous research was lacking and its postmortem processes were largely undocumented except for the publication by Lasczkowski *et al.,* (2002).

Most of the techniques applied during this research are not quantifiable or empirical but rely on qualitative, observational and specialised experience on behalf of the researcher. However, this does not make the work any less valid or scientific. Consequently, with future work and refinement, the application of this work in a forensic setting could fulfil the requirements of any expert witness / testimony for example the American guidelines pertaining to the Daubert laws of evidence. These laws state that the method of analysis utilised should be tested, peer reviewed and published, with known or potential error rates and be accepted by the scientific community (Grivas and Komar, 2008).

Chapter 8: References

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Web based programs used:

NCBI BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi

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Chapter 9: Appendices

Appendix 1: The Animal By-Products Regulations 2005

Approval of the Use of Animal By-Products for Research Purposes

1) In accordance with Article 23 of Regulation (EC) No 1774/2002 the Secretary of State approves -

Name of Operator	**Mr Raul Sutton**
Address of authorised premises	**University of Wolverhampton's Crop Research Facility** Compton Park Campus Compton Road West Wolverhampton WV3 9DX

to bury parts of pig carcases on the above premises for the purposes of researching into their decomposition.

2) Category 2 material may be used.

3) All burial sites must be marked and burials recorded (map references and dates of Burial) and notified to Leicester AHDO;

4) All burials are contained within cages which must be secured to prevent access by Scavengers (carnivores and vermin);

5) Measures to avoid access by insects must be employed.

6) Any unused material, or material recovered from the ground, must be disposed of in accordance with the Animal By-Products Regulations 2005, ie to incinerator approved to receive this material, or to an approved renderer;

7) There must be no livestock co-located on the premises used for burials;

8) Details of porcine material received/purchased is recorded with date of purchase, quantity, and source. (These records must be kept for a period of 2 years.)

Appendix 2: Risk Assessment for Compton Campus

Risk assessment of activities at the Compton site, Wolverhampton

Hazard	Degree of Potential Risk to Investigative Persons	Mitigation	Degree of Risk after Mitigation
Risk of infectious agents – pathogens, decomposing carcasses. Soil microbes.	High	Protective clothing, Gloves, face mask if required. Tetanus vaccination.	Medium - Low
Risk of injury at site, tripping, contact with sharp objects (stones, broken glass).	Medium	Perform all acts with due care. Inform on site technicians of work dates and times. Be aware of first aid precautions and location of first aid equipment.	Low
Risk of assault	Medium - low	Travel to and from site during daylight. Use shuttle bus if possible. Don't work at the site at night.	Low
Incorrect use of equipment.	Medium	Follow health and safety precautions while handling sharp equipment such as scalpels.	low
Flora	Low	Wear gloves when handling all unknown plant species.	Low
Insects	Low	Beware of hazardous insects. Wear gloves when handling insects.	Low
Climate	Low	Wear the appropriate level of clothing during the winter. Take regular breaks. During the summer apply sun screen.	low

Appendix 3: Fungal DNA sequences

There are two sequences per sample. 1 sequence with the ITS1 primer and another with the ITS4 primer.

Sample 1: *Aspergillus fumigatus*

TTTACAAGGAGGAGGGGCCATTCTTGGGGTTCAACCTCCCACCCGTGTCTATCGTACCTTG
TTGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAGGCCCTGCGCCCCCGGGCCCG
CGCCCGCCGAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCTGAGTTGATTAT
CGTAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGC
AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACG
CACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCA
AGCACGGCTTGTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCAG
CGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGTAGGCCCG
GCCGGCGCCAGCCGACACCCAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGA
TACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAATCATTACCGAGTGAGGGCCCTCT
GGGTCAACCTCCACCCGTGTCTATCGTACCTTTGTTGCTTTGGCGGGCCGCCGTTTTCGAC
GGCCGCCGGGGAGGCCCTGCGCCCCCGGGCCGCGCCGCCGAAGACCCCAACATGAACGC
TGTTCTGAAAGTATGCATTCTGCAGTTGATTTATTCGTAAATCAGTTAAAACTTTTCAACA
ACGAATCTCTTGGGTTCGTCTTCATGAAAGACGCGCAAGTGCCATGAAATAGATGTGAAT
TTGCATAATTCGGTGAATCCTCTATTCTTGAAACGCCATCGCGCCGTAGAATGCCGGGGT

Sample 2: *Aspergillus fumigatus*

TGGGAGGGGGTTCTACCTGATCGAGGTCACCTTAGAAAAATAAAGTTGGGTGTCGGCTGG
CGCCGGCCGGGCCTACAGAGCAGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGG
TGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGAGAGGGGGACGGGGGCCCAACACACA
AGCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGG
GCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCG
CATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACT
GATTACGATAATCAACTCAGACTGCATACTTTCAGAACAGCGTTCATGTTGGGGTCTTCG
GCGGGCGCGGGCCCGGGGGCGCAGGGCCTCCCCGGCGGCCGTCGAAACGGCGGGCCCGC
CGAAGCAACAAGGTACGATAGACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCG
GTAATGATCCTTCCGCAGGTACCCTTACGGAAGCATTATCGAGTGAGTGGCCCTCTGGGT
GCGACCTCCCCGTGTCTAGTGACTTGTTGCTTCGGCGGGCGGCGTTTCACGGCGCCGGGG
AGGCCTGCGCCCCAGGGCCGCGCCGCCGAAGGACCACATGAACGCTGTTCTGAAAGTAT
GCGGTCTGAGTTTGATTATCGTAATCAGTTTAAAACTTTCAACAAGGAATCTTGGTTCGCA
TCATGAAAAAGCAGCACAGGTGGCATAGTAATGTGAGTTGGCAGAAATCAGTGAAATCA
TCGAGTCTTTGAACACAGTCGCGCCGGTTATTTGGGGGGGGGGAGGCCCGCCCACATCGT
CGCGTCCGTGTTTTGTGGAGGCCGTCCCCCTCCCCTCCAGGGGAAAATCATCAAAAAGAT
GGGTCGTCCCCCTCTCGCCTCTCGAGAGAGAGGGGGGTGTGTGTCCTCTCTGTGTAAGTC
GCGCGGCGCCCCACGCGCCCATATTATTTTTGTGTGTGGAGACGCGAGAGAGATGAGGAG
TAGTCACCACCTATATATACGCTACGGAGAGAGAGGAGAAAAAACTC

Sample 3: *Aspergillus fumigatus*

GGAAACCGAAGATGGAGGGACATCATGGGTCAACCTCCCACCCGTGTCTATCGTACCTTG
TTGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAGGCCCTGCGCCCCCGGGCCCG
CGCCCGCCGAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCTGAGTTGATTAT
CGTAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCA
GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGC
ACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAA
GCACGGCTTGTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCAGC
GGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGTAGGCCCGG

CCGGCGCCAGCCGACACCCAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGAT
ACCCGCTGAACTTAAGCATATCAAAAGCCGGAAGAATCATTACCGAGTGAGGGCCCTCTG
GGTCAACCTCCACCCGTGTCTATCGTACTTGTTGCTTCGGCGGGCCGCGTTTCGACGGCCG
CCGGGGAGGCCTGCGCCCCGGGCCGCGCCCGCCGAAGACCCAACATGAACGCTGTTCTG
AAAGTATGCGTTCTGAGTTGATTTATCGTAAATCGTTAAAACTTTCAACAACGATCTCTTG
GGTTGCGCAGTCAGTGAAGGAAGCGCAAAATGCATAAGTGAAGTGTGAATTGCAGTAAT
TCAGTGATTCATCGAGTCTTTGAACGCATCCGTGTGTAGTGCGCGGGGCTGAGCGTATCG
CTCTCTCGCGACGCTGAGTCTTCCCGGGCCGCACGCCCAAAGCAACGCCACCTCCGTCTC
AAAGAGTGGGGTGTTGTCCCGTCTTTAAGCAGCGCGCCGCGACACCCAATTTATTTTCTT
GGGGTACCCCGTAGAGGGGGGAACCGCGTAACTAGACTCTCTAGCGGCAGAAGAGAAAA
AA

Sample 4: *Aspergillus fumigatus*

TGTAGGGGTCTACCTGATCCGAGGTCAACCTTAGAAAAATAAAGTTGGGTGTCGGCTGGC
GCCGGCCGGGCCTACAGAGCAGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGT
GCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGAGAGGGGGACGGGGGCCCAACACACAA
GCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGG
CGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGC
ATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTG
ATTACGATAATCAACTCAGACTGCATACTTTCAGAACAGCGTTCATGTTGGGGTCTTCGG
CGGGCGCGGGCCCGGGGGCGCAGGGCCTCCCCGGCGGCCGTCGAAACGGCGGGCCCGCC
GAAGCAACAAGGTACGATAGACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCGG
TAATGATCCTTCCGCAGGCACCCTACCGGAAGGTTATGAAGTGAGTGGCCGTCTGGGTGC
GACCTCCACCGTGTCTATGTGACTAGTGTGCTTCGCTGGTCGCCGTGTGACGGCCGCCTGG
GAGGCCTGCGCCCCAGGCCCGCGCCCGCCGAAGACCCAACATGAACGCTGTTCTGAAAG
TATGCGCTCGCAGTTGCATTATCGTAGATCAGTGTAAAACTTTCACAAGGATCTTGGTTCG
CAGTCATCAAGGACGCGCGGAGTCATCAGGTGAGATGCTGTATTGCACAGGTCATGAGAT
CATGAGTCGTTTGACACATCGCGCGGTCATCGGGGAGGGCGAGCGGCGCAACTCGTGGTC
GCTCTGTAGTTCGCGGGCGACTGCCCCAAGAAGTGAACCGTCGTCCGAGAGAGAGGGGG
TTGTCCCCTCTTTAGCGAGGGCACGCAACCACATTTGTCGGGGGTTAGCACGCAGCGGTA
GGTATACCTCACTTAGCATACAGCGCGAG

Sample 5: *Penicillium*

TCGGAAAATTGAGGGGCACTCCTGGGTCCACCTCCCACCCGTGTTTATTTTACCTTGTTGC
TTCGGCGGGCCCGCCTTAACTGGCCGCCGGGGGGCTTACGCCCCCGGGCCCGCGCCCGCC
GAAGACACCCTCGAACTCTGTCTGAAGATTGTAGTCTGAGTGAAAATATAAATTATTTAA
AACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT
ACGTAATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCC
TGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCACGGCTTGTGTG
TTGGGCCCCGTCCTCCGATCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTC
CGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGA
TCAACCCAAATTTTTATCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA
GCATATCAATAAGCGGAGGAATACCCAGTGAGGGCCCTCTGGGTCAACTCCCCGTGTTTT
ATTTTACTTGTTTGCTTTGGGGGGGCCCTTAACTGGTCCCGGGGGGGGGCTTTACGCCCGG
GCCGCGCCCCCAAAAACCCTCCAACTCTGATCTGAACAAATGTATCCTGAAGTGAAAAA
TATAAATTTATTC

Sample 6: *Penicillium*

TCCGAGGTGGGGGTCTTCTGGTCCGAGGTCACCTGCGATGAAAATTTGGGTTGATCGGCA
AGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCGGACGC
GGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGATCGGAGGACGGGGCCCAACACACA
AGCCGTGCTTGAGGGCAGAAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGG
GCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTTGCAATTCACATTACGTATCG
CATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAAAT
AATTTATATTTTCACTCAGACTACAATCTTCAGACAGAGTTCGAGGGTGTCTTCGGCGGGC
GCGGGCCCGGGGGCGTAAGCCCCCCGGCGGCCAGTTAAGGCGGGCCCGCCGAAGCAACA
AGGTAAAATAAACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCGGTAATGATCC
TTCCGCAGGCACCCCTAACACGAGGGAAACGATTGAGGGCCCTGGGGTCAACGTCCACC
CGTGTTTATCTTGCGTTGTTGCTTCGGGGCGCTTAACTGGCCCGCGGGGGCTTACGCCGGG
CGCGCGCCGCGAAACACCTCGAACTCTGTCTGAAGATGTAGTCTGCAGTGAAAATATAAA
TTATTTTAAAACTTTCACAACGGATCTTGGTTCGCATGATCAAGAAAGCAGCGCGTGCAT
ACTGAAGTGTGAATTGCAAATTCAGTGAATTCATCAGTTCTTTGTACGCACTGCCCTTTCC
GGGGGGGGGAGGCGTGACCCAGTCATCCTGTGTGTTGTGTGGGCCCCCTCCATCCCGGGA
GAAGGGGCGCAAGGCAGCGCGCTCTGCTCCCGTCCCTCGAGCGGGATGGGGCTGTGTCC
ACCGTCTTTAGAGCCGGCCGCGCCTTGCGCGATTACCCAAATTTGTATTCTCCGGTTAACA
CCTCGCGATAGGGTGGGGAGTACCCGCTAAATTTAAAGCAGTAGTTGTGGCGGGAGGAG
AGAGAAAACA

Sample 7: *Aspergillus fumigatus*

CCAAAAGAAGGGGGGGCAATCCTGGGGTCCACCCTCCCACCCGTGTCTATCGTACCTTGT
TGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAGGCCTTGCGCCCCCGGGCCCGC
GCCCGCCGAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCTGAGTTGATTATC
GTAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAG
CGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCA
CATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAG
CACGGCTTGTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCAGCG
GCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGTAGGCCCGGC
CGGCGCCAGCCGACACCCAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGATA
CCCGCTGAACTTAAGCATATCAATAAGCGGAGGAATCATTACCGAGTGAGGGCCCTCTGG
GTCCACCTCCACCGTGTCTATCGTAACTTGTTGCTTCGCGGGCCCCGTTTCGACAGCGCCG
GGGAGGCTTGCCCCCGGGCCGCCCCGCCGAAACCCCACAAGAACGCTGTTTCGAAAAGT
ATGCCTCGATTGAATTATCAAAATCAGTTTAAAAACTTTCACACGCAATCTCTGGGCTGTC
TCTCATAAAAGGAGCACAAAAGCAATTAAACTACATGGTGAAATTGCAAAATCCAGTGA
CTCTGCAGCTCTATTGAAACGCCCTTGCCTTTGTGGGGGGGGGCGGGGAG

Sample 8: *Aspergillus fumigatus*

ACCGAAAGGGGTTCAACCCTTAAGAAAAATAAAAGTTGGGTGTCGGCTGGCGCCGGCCG
GGCCTACAGAGCAGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGC
TGCCTTTCGGGCCCGTCCCCCGGGAGAGGGGGACGGGGGCCCAACACACAAGCCGTGCT
TGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGT
GCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCT
GCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTGATTACGA
TAATCAACTCAGACTGCATACTTTCAGAACAGCGTTCATGTTGGGGTCTTCGGCGGGCGC
GGGCCCGGGGGCGCAAGGCCTCCCCGGCGGCCGTCGAAACGGCGGGCCCGCCGAAGCAA
CAAGGTACGATAGACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCGGTAATGAT
CCTTCCGCAGGTTCACCTGCGGAAGGTTCACTAGCGTGGGCGGGTGGAACAGCTCCACCG
AGTCATCGTACTTTTTGTTTTAGGGAGGCCCGTTTTCACAGCCGTGGGAGGTCTTGCCGCC

GGGGCCTCCCTCCAAAGCTCCACAGAGGCGGTTCCGCAAATTAGAGATCGAAGCTCGAAT
TTACGTT

Sample 9: *Penicillium*

ACCGAAAGGGAAGGGCCATCTGGGTCAACCTCCCACCCGTGTTTATTTTACCTTGTTGCTT
CGGCGGGCCCGCCTTAACTGGCCGCCGGGGGGCTTACGCCCCCGGGCCCGCGCCCGCCG
AACACACCCTCCAACTCTGTCTGAAGATTGAAGTCTGAGTGAAAATATAAATTATTTAAA
ACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAACGAAATGCGATAC
GTAATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTG
GTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTT
GGGGCCCGTTGCCCCCCCGCCCCGCTCTCGAGGGGAAAGGGCCCGAAATCAGGCGACGC
GCCCCCCCGCGTCCTCGACCTTGTGGGGCTTTCTCACCGGCTGTGAAGGCCCGGCCGGCG
CCCCCCAACCTCCAACTTTGTTTTTCCAACGTTGAGGACGAATAGTGTATGGATACCCGCT
AATCTTTCGCATATCAATACCCGAAGGAAGTGTGGCGCGTGACGGCACAATCTGCAAACC
CGCGGCAGAGGTCGCATACCGCCCTGGGGACGTCATCGCGGGTGGAGAGGCACACCGTG
CGCTTATAAATCGTGTGAGGGG

Sample 10: *Penicillium*

ATGCTCCTACCTGATCCGAGGTCACCTGGATAAAAATTTGGGTTGATCGGCAAGCGCCGG
CCGGGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGC
CGCTGCCTTTCGGGCCCGTCCCCCGGAGATCGGGGGACGGGGCCCAACACACAAGCCGG
GCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAA
TGTGCGTTCAAAGACTCGATGATTCACTGAATTTGCAATTCACATTACGTATCGCATTTCG
CTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAAATAATTTAT
ATTTTCACTCAGACTTCAATCTTCAGACAGAGTTCGAGGGTGTCTTCGGCGGGCGCGGGC
CCGGGGGCGTAAGCCCCCCGGCGGCCAGTTAAGGCGGGCCCGCCGAAGCAACAAGGTAA
AATAAACACGGGTGGGGAAGGTTGGAACCCAGAGGGCCCTCACTCGGTAATGATCCTTC
CGCAGGTCACCCCCGACGGATGACCGAGGAGAAGGCCTCTGGGTCAACCTCCCCCCCTGT
TTATTTTACTTGTTTGCTTCGCGGGCGCTTAACTGGCGCCGGGGGCTTACGCCCGGGGCGC
GCCCGCCGAAAACACCTCGAACTCTGTCTGAAGATGAAAGTCTGAGTGAAAATATAATTA
TTTTAAAACTTTTCACACGGATCTTGGGTTTCGCATCATAAAAAAGCACTCAGTGCATACA
TAATGTGAATTGCAATTCACTGAATCATCGAGTCGTTTGAACGCAATCGGCGGGCCTCTA
GTTCCGGGTGGGGCATGGCTGGTCCAGCAGCCTCTCTCGCCGCCGCTGGTGTGTGTGTGC
CGCCACCCTCCTACCACTCTCTCGGGAGAACGGGCTCACACCCGTCTCGTCGGACGGTGT
GCGCTTGTGTCCCCCGTCGTAGGCCCAGGCCCGCGCCTAGCGCATAAACCCAAATTCTGA
TCCCGGTAACCTCCGATCAGGTCGGATACCGCTACTAGCTGCAGCTCCAGCCACCAGATG
TCGCGCTTTCACCTCCGGGCGCAGATGCATCCAC

Sample 11: *Aspergillus fumigatus*

GGGTTACGGAGATGAGGGACATCTGGGTCCACCTCCCACCCGTGTCTATCGTACCTTGTT
GCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAGGCCCTGCGCCCCCGGGCCCGCG
CCCGCCGAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCTGAGTTGATTATCG
TAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC
GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCAC
ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGC
ACGGCTTGTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCAGCGG
CGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGTAGGCCCGGCC
GGCGCCAGCCGACACCCAACTTTATTTTCTAAGGTTGACCTCGGATCAGGTAGGGATAC
CCGCTGAACTTAAGCATATCAAAACCCCGGAAAGAATCATTACCGAGTGAGGGCCCTCTG
GGTCCAACCTCCACCGTGTCTATCGTACTTGTTGCTTCGGCGGGCCGCGTTTCGACGGCCG
CCGGGGAGGCCCTGCGCCCCGGGCCGCGCCCGCCGAAGACCCAACATGAACGCTGTTCT

GAAAGTATGCAGTCTGAGTTGATTATCGTAATCAGTTAAAACTTTCACAACGGATCTCTT
GGTTCGCATCATGAAGAACGCAGCGAAATGGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAAATCATCGAGTCTTTGAACGCACATTGGCGCGCTTATTTCGGGGGGCCATGCCTGT
CGAGCCCTCGGAGCGCCGCCTGGTGTATGGTGACCTCTCTCCGGGGGAGAGAGCCAAAA
GGCAGGCGACACGAGTCAGTGCTTTTCCCGTCTGTTGTCGCGACGCGCCACAACACATTA
TATTTGTGGTCTCGCCCCGACGGGGGGAGACCTCTAATATATATTGTGCGAGGAGAAGA

Sample 12: *Aspergillus fumigatus*

CTGATCTACCTGATCGAGGTCACCTTAGAAAATAAAGTTGGGTGTCGGCTGGCGCCGGCC
GGGCCTACAGAGCAGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCG
CTGCCTTTCGGGCCCGTCCCCCGGGAGAGGGGGACGGGGGCCCAACACACAAGCCGTGC
TTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATG
TGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGC
TGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTGATTACG
ATAATCAACTCAGACTGCATACTTTCAGAACAGCGTTCATGTTGGGGTCTTCGGCGGGCG
CGGGCCCGGGGGCGCAGGGCCTCCCCGGCGGCCGTCGAAACGGCGGGCCCGCCGAAGCA
ACAAGGTACGATAGACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCGGTAATGA
TCCTTCCGCAGGTTCACCTACGGAATCATTACCGAGTGAGGGCCCTCTGGGTCCAACCTC
CACCCGTGTCTATCGTACCTTGTTGCTTCGGCGGGCCGCCGTTTCGACGGCCGCCGGGGA
GGCCCTGCGCCCCGGGCCCGCGCCCGCCGAAGACCCAACATGAACGCTGTTCTGAAAGTA
TGCGTCTGAGTTGATTATCGTAATCAGTTAAAACTTTCACAACGATCTCTTGGTTGCGCAG
TCAGTAAGAACGAGCAAAAATCATAAGCTGAGTGTGAATTGCAGAATTCGTGAAATCAT
CAGTCTTTTGAACGCCATCGTGCTGGTACTTCCGGGGGCATGGCGGCTCAGCGCTCTCGTC
GCGCGTGTTTCTCTTCGCGCGGCCACGGCCCGAAGGACGGCGGCCCGCGTCTCGGTTCCT
CGAGAGAGAGGGGGCGTTGTTCCTGTCTCGTGTAGGCGAGCCGCGCCGCGCGACCCACC
AATTGTCTAGGTGGACCTCGGATCCGGTAGGTACCCTGACTTAGCTGTTGCGGGGGGAGA
GAGTAGTAT

Sample 13: *Aspergillus fumigatus*

GGGTTTCGGGGGAGGAGAGCATCTGGGGTCACCTCCCACCCGTGTCTATCGTACCTTGTT
GCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAGGCCCTGCGCCCCCGGGCCCGCG
CCCGCCGAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCTGAGTTGATTATCG
TAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC
GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCAC
ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGC
ACGGCTTGTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCAGCGG
CGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGTAGGCCCGGCC
GGCGCCAGCCGACACCCAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGATAC
CCGCTGAACTTAAGCATATCAAAGCCGGGGAGAGGATCATTACCGAGTGAGGGCCCTCT
GGGTCCAACCTCCACCCGTGTCTATCGTACCTTGTTGCTTGGCGGGCCGCCGTTTCGACGG
CCGCCGGGGAGGCCCTGCGCCCCCGGGCCGCGCCCGCGAAGACCCAACATGAACGCTGT
TCTGAAAGTATGCGTCTGAGTTGATTATCGTAATCAGTTAAAACTTTCACAGCGCATCTCT
TGGTTGCGCAGTCATCAAGGAACCACAAAGTCATAGATTAAGTGTGAAATTTGCAGAATT
TCAGTGAAATCATCAGTCTTTGACGCAGTCTGCCCTAGTTCCGGGGGGGCATGCCGGACA
GACTCGCTCGCTCGCGTGTGTCGACTCTCCCGCGGGAACGGGCCCAAGGCACGGCGGCAC
CCGTTCGTCCTCGAACGTAGTGGGGCTTTTGTTCCTGCTCTGTTAGGCCAGCGCAGCCGAA
CCCAACTTTATTTGTTAGGTTGGACTCGGATTCGGTAGAGATACGCCGTGAACTTAGCTTA
CATCTCGCGAG

Sample 14: *Aspergillus fumigatus*

TGGACGGGATCCTACTGATCCGAGGTCACCTTAGAAAAATAAAGTTGGGTGTCGGCTGGC
GCCGGCCGGGCCTACAGAGCAGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGT
GCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGAGAGGGGGACGGGGGCCCAACACACAA
GCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGG
CGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGC
ATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTG
ATTACGATAATCAACTCAGACTGCATACTTTCAGAACAGCGTTCATGTTGGGGTCTTCGG
CGGGCGCGGGCCCGGGGGCGCAGGGCCTCCCCGGCGGCCGTCGAAACGGCGGGCCCGCC
GAAGCAACAAGGTACGATAGACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCGG
TAATGATCCTTCCGCAGGCCCCTCAAAGGAGAGAGATTACGAGTGGGGGCCGTCTGGGGT
GCGAACTCACCCGTGTCTATGTACTAGTGTGCTTCGTGGGCCGCCGTTTCGACGGCCGCC
GGGGAGGCCTGCGCCCCCGGCCCGCGCCCGCCGAAGACCCACATGAACGCTGTTCTGAA
AGTATGCACTCTGAGTTGATTATCGTAATCAGGTTAAAACTTTCACAACGATCTTGGTTCG
CGTCATTAAAAAGCGCAACGCGATAATGATGTGGAATTGCAGACTCGTGAATCATGGAGT
CTTTGAACGCCAGTTCGCGTTATGCGCGGGGGCCAGTGCGAGTCGCACATCGAGAGACGG
CTGGGTGTTGGGGCCCTCTCTTCGCGGAGGAGAGACCAAAGAACCGTCCAGTCCGTCTCG
AGAGAGGGGGGTTTGTGCTCTCTCTCTCTGAGCCCGACGCCCCAGCGACTACTTATTGTTC
GTGTACACCGGAACGGTGGAGGACCCCAATTTAGCCTGTCGGGGGAGAAAATAGA

Sample 15: *Aspergillus fumigatus*

GGGAAAAGAAGAGAGGGACTCTGGGTCACCTCCCACCCGTGTCTATCGTACCTTGTTGCT
TCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAGGCCCTGCGCCCCCGGGCCCGCGCCC
GCCGAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCTGAGTTGATTATCGTAA
TCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATT
GCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACG
GCTTGTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCAGCGGCGG
CACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGTAGGCCCGGCCGGC
GCCAGCCGACACCCAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCG
CTGAACTTAAGCATATCATAAGCCCGGAGGGAATCATTACCGAGTGAGGGCCCTCTGGGT
CCAACCTCACCGTGTCTATCGTACTTGTTGCTTCGCGGCGCCGTTTCGACGGCCGCCGGGG
AGGCCTGCGCCCCGGGCCGCGCCGCGAAACCCACATGAACGCTGTTCTGAAAGTATGCA
ATCTGAGATTGATTATCGTAATCAGTTAAAACTTTCAACAACGCATCTCTTGGTTCGCATC
ATTGAGAAGACAAGAGTCATAAACTAATGTGAATTGCAGATTCATGAAATCATCAGTCAT
TGAACGCACATCTGCCTATGGCCGGGGCATGCGCTCGCCCTGCGTCGCGAGTGTGAGTGG
GCCCGCCCTCTCGCGCGGAGGGCGCGAAGAGACGCCACCGCCCCGCCGCTCCCGAGATG
GGGGTGTCGTCTACCCTCTGTGAGCGCGCGGCGCGCCGCAGACCAACTTTGTCGTCTAGA
GGTGACTCGCGAGGAGGAGGAGGATCCAGCGTAATTAGCAGTATGGGAGGAGTGGAAGA
AT

Sample 16: *Aspergillus fumigatus*

TTCGAATGGGAGCTACTGATCGAGGTCACCTTAGAAAATAAAGTTGGGTGTCGGCTGGCG
CCGGCCGGGGCTACAGAGCAGGTGAGAAGCCCCATACGCTCGAGGACCGGACGCGGTGC
CGCCGCTGCCTTTCGGGCCCGTCCCCCGGGAGAGGGGGACGGGGGCCCAACACACAAGC
CGTGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCG
CAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCAT
TTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTGAT
TACGATAATCAACTCAGACTGCATACTTTCAGAACAGCGTTCATGTTGGGGTCTTCGGCG
GGCGCGGGCCCGGGGGCGCAGGGCCTCCCCGGCGGCCGTCGAAACGGCGGGCCCGCCGA
AGCAACAAGGTACGATAGACACGGGTGGGGAGGTTGGACCCAGAGGGCCCTCACTCGGT

AATGATCCTTCCGCAGGCACCCCCCAAAGGGAGGGTCCGAGGTGAGGGCCTCTGGGTCC
AACTCACCGTGTCTATGCACTTGTTGCTTGGCGGGACGCCGTTTCGACCGCCGCGGGGAG
GACTGCGCCCCGGCCGCGCCGCGAAAACCCACATGAACGCTGTTCTGAAAGTATGCACTC
GCAGATTGATTATCGTAGATCAGCTTAAAACTTTCACACGGGCTTGGTTCGCGTCATGAA
GAAGGACAAGGCATCGGTGATGTGAATTGCACAGGTCGTGAATCACGAGTCTTGAACGC
CACTCCGCCGTGATTCGGGGGAAAGGCGACGCGTTTCGTGCGGGTGGTATGTGCGGCGGC
CACCCTCCAAGGTGTGGTCACCTCTCACGTCGTGCGAGGCGCGGCGCTCTCCAAATAGAT
GGTGTGTGTGTCTCCCTCGTGAAGCGAGACGGCAGTAGACACCAAATTGTGTGGTAGAAG
TAGCACGCAGAGAGGGGAGTACTACCCTTAACGTAAGTGAGGGGAGAGAGAGAGA

Appendix 4: Hyperladder 1